Investigating the breakdown of chemical control in brown planthoppers from Asia

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

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Abstract

The brown planthopper (*Nilaparvata lugens* Stål) is one of the most economically important pests of rice across Asia. The control of this pest has mainly relied upon the use of insecticides. However, rice ecosystems across Asia are being put at severe risk due to the over-reliance on certain insecticides, mainly imidacloprid (neonicotinoid) and ethiprole (phenylpyrazole), which the pest is now showing widespread resistance against. The evolution of resistance represents a tangible threat to the long-term sustainable control of this species.

Field strains were placed under selection separately with imidacloprid and ethiprole, leading to increased resistance within these strains. Selection with ethiprole was demonstrated to cause cross-resistance to another phenylpyrazole, fipronil. A *de novo* transcriptome was generated and was used to search for differentially expressed genes between susceptible and insecticide resistant populations. This transcriptome also allowed assembly of insecticide target sites, that were then screened for mutations.

The most recent class of insecticide to show decreased efficacy against the brown planthopper was the phenylpyrazoles (Fiproles). Potential mechanisms for resistance, both metabolic and target site were studied with the use of a model organism, *Drosophila melanogaster*. These have implicated a mutation, A301S, in the *Rdl* channel in ethiprole resistance, but not in causing significant fipronil resistance.

Point mutations that occurs at the target site for imidacloprid and previously linked with resistance, were not witnessed in the field strains monitored for this PhD. However, it was discovered that a single cytochrome P450 gene (*CYP6ER1*) was markedly overexpressed in all the imidacloprid resistant strains tested. This gene displayed considerable coding sequence variation between the susceptible and resistant strains. Of the eight *CYP6ER1* variants found, two were highly expressed. Studies *in vivo* showed these *CYP6ER1* variants conferred significant resistance to imidacloprid compared to a *CYP6ER1* variant from susceptible *N. lugens*. It was concluded that coding sequence changes in *CYP6ER1* were the primary role for imidacloprid resistance, with overexpression contributing in a secondary role.

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Abbreviations

AA	Amino Acid
ABC transporter	ATP binding cassette transporter
ABT	1-aminobenzotriazole
ACh	Acetylcholine
AChE	Acetylcholinesterase
AI	Active ingredient
bp	Base pair(s)
C	Celsius
CCE	Carboxylesterases
cDNA	complementary deoxyribonucleic acid
CDS	Coding sequence
CL	Confidence interval
cm	Centimetre
CYP	Cytochrome P450-dependent monooxygenase
DDT	Dichlorodiphenyltrichloroethane
DEF	S.S.S-tributyl phosphorotrithioate
DEM	Diethyl maleate
DEseq2	Differential gene expression sequencing analysis 2
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleoside triphosphates
EdgeR	Empirical analysis of digital gene expression data
Et-Br	Ethidium bromide
FastQC	Fast quality control
g	Relative centrifugation force (g-force)
g	Gram
GABA	*gamma*-aminobutyric acid
gDNA	genomic deoxyribonucleic acid
GluCl	Glutamate-gated chloride channel
GO	Gene onthology
GOI	Gene(s) of interest
GST	Glutathione S transferases
h	Hour
IPM	Integrated Pest Management
IRAC	Insecticide Resistance Action Committee
IRRI	International Rice Research Institute
kdr	Knockdown resistance
L	Litre
LC	Lethal concentration
LD	Lethal dose
mg	Milligram
min	Minute
mL	millilitre
mM	Millimolar

µg	Microgram
µL	microliter
nAChR	Nicotinic acetylcholine receptor
ng	Nanogram
nt	Nucleotide
ORF	Open reading frame
P450s	Cytochrome P450-dependent monooxygenase(s)
PBO	Piperonyl butoxide
PCR	Polymerase chain reaction
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
Rdl	Resistant to dieldrin
RNA	ribonucleic acid
RR	Resistance ratio
s	Second
SC	Suspension concentrate
SE	Standard error
SNP(s)	Single nucleotide polymorphism(s)
SRS	Substrate recognition site
Taq polymerase	*Thermus aquaticus* polymerase
TGAC	The Genome Analysis Centre (Norwich)
Tm	Melting temperature
TM	Transmembrane domain
VGSC	Voltage gated sodium channel

Chapter I Introduction

1.1 Global food security

The task of providing worldwide food security is one of the greatest challenges facing humanity in the 21st century. The necessity to increase global food production to feed an ever-expanding population, but to do so more sustainably than currently, is an incredibly complex challenge. The current global population is 7.3 billion and there is a well-documented prediction that it will rise by a further 2 billion in the next 30 years. However, even now there are approximately 790 million malnourished people, with inadequate access to protein and micronutrients (FAOSTAT, 2017b). A host of factors are threatening global food security; these include the impact of climate change and the increase in competition for land, water and energy use (Godfray *et al.*, 2010). The growing demand for meat and dairy products from rapidly developing countries such as China is also putting pressure on food production systems (The Royal Society, 2009).

One strategy proposed to tackle this issue has been sustainable intensification (The Royal Society, 2009). This is generally described as producing more food from the land than currently, but at the same time reducing the environmental impact of modern agriculture (Godfray *et al.*, 2010). Part of this will involve closing the yield gap. Godfray et al. describe this as the disparity between the yields that are theoretically possible from a combination of inputs and the production that is physically realised. There are many reasons for this gap, ranging from biotic

factors such as weeds, fungus and insect pests to abiotic factors including, water, soil quality, sunlight and salinity.

The main grain crops (maize, rice, wheat and barley cereals) are the most intensively produced crops in the world. Of these rice is estimated to be the major staple for half the world's population. The advent of the green revolution in the 1960s led to a large increase in rice yields, primarily through the use of high yielding rice varieties, synthetic fertiliser and pesticide application (Bottrell and Schoenly, 2012). In 2014 there were 741 million tonnes of rice produced, second in volume only to maize (FAOSTAT, 2017a). This rice was produced at an average of 4.5 tonnes per hectare, well below the theoretical average of 8.5 tonnes per hectare (Cassman, 1999). A major factor for this disparity between actual and potential yield was due to the impact of insect pests.

1.2 Insect pests of rice

Rice is targeted by more than 100 species of insects, and within this there are approximately 20 pest species that are capable of inflicting damage above economic thresholds (Pathak and Khan, 1994). Classified within this group are the stem borers, planthoppers, leafhoppers and gall midges. With the advent of the green revolution and a shift to high yielding cultivars of rice there was a significant shift in associated pest damage. Species such as brown planthopper, white-backed planthopper and leaffolders had been relatively minor pests compared to the stem borers, but became major rice pests with the introduction of high yielding rice varieties (Pathak and Khan, 1994).

1.3 *Nilaparvata lugens*

The brown planthopper, *Nilaparvata lugens* Stål (Hemiptera: Delphacidae), is today one of the most significant pests of rice throughout Asia, causing yield and economic losses. *N. lugens* (Fig.1.1A) is a monophagous herbivore and can limit yield potential in the rice crop through two mechanisms. Firstly, it is a sucking pest and causes nutrient depletion in the plant via direct phloem-sap feeding. When levels of *N. lugens* reach high infestation levels this feeding can cause a series of deleterious effects known as 'hopperburn' (Gorman *et al.*, 2008). This phenomenon is visualised by characteristic stunting, wilting and browning of the crop (Fig. 1.1B). Secondly, *N. lugens* also acts as an efficient vector for various rice pathogens, including rice ragged stunt virus and rice stripe cereal viruses (Cabauatan, Cabunagan and Choi, 2009). The combination of these two effects causes rice yield to be substantially reduced (Cheng, 2009).

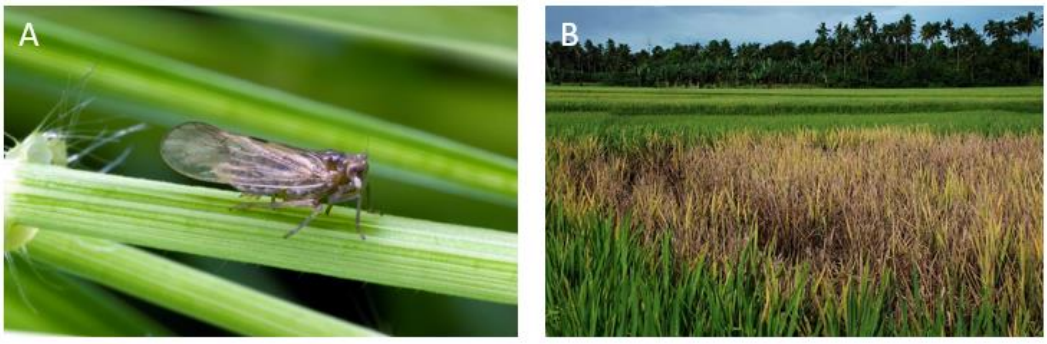


Fig. 1.1 A) An adult brown planthopper, *Nilaparvata lugens*. B) 'Hopperburn'.

The earliest reputed outbreak of *N. lugens* was recorded in circa AD 18 in Korea (Dyck and Thomas, 1979), with further outbreaks documented in AD 697 or 701 in Japan (Grist and Lever, 1969). Since *N. lugens* was not taxonomically defined until 1854 (Dupo and Barrion, 2009), it is not possible to confirm these early reported

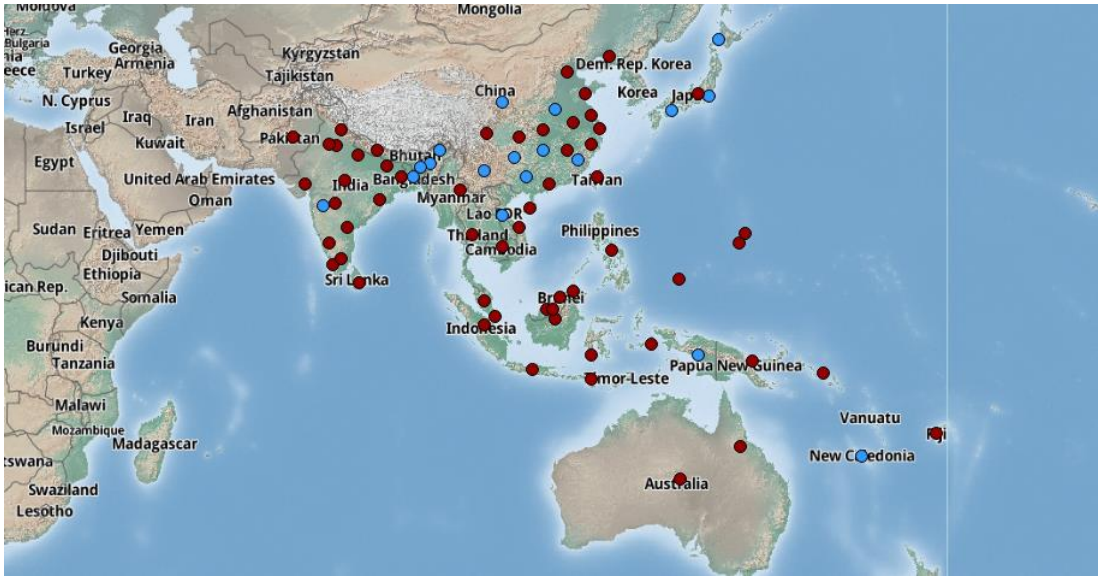
incidents as being definitively *N. lugens*. During the 1960s, with the implementation of green revolution technologies for rice production, *N. lugens* became a key threat to rice production (Bottrell and Schoenly, 2012). Before the 1960s there had been sporadic recorded outbreaks of *N. lugens*, usually in temperate areas, such as Japan and Korea (Dyck and Thomas, 1979), but it was not regarded as a major pest of rice during this time.

The life cycle of *N. lugens* is divided into three distinct phases: egg, nymph and adult. The eggs are laid directly into the tissue of the lower part of the rice plant, predominantly into the leaf sheaths (Mochida and Okada, 1979). Depending on temperature the egg phase lasts 7-11 days in the tropics of Asia. The nymphal phase, which consists of 5 development stages - from 1st to 5th instars, lasts approximately 10-15 days (Mochida and Okada, 1979). Mochida and Okada also record that at 25^{o}C the time between emergence of an adult in one generation to that of the subsequent generation is 28-32 days. However, at higher temperatures (28^{o}C) this can be reduced to as little as 23-25 days. This variation in development time is reflected in the number of generations per year recorded in the tropics compared with temperate regions. The tropic regions can host 12 generations per year (Dyck *et al.*, 1979), whilst temperate regions can only sustain up to 3 generations of *N. lugens* per year (Perfect and Cook, 1994).

Short generation times is one the key factors that makes *N. lugens* such a damaging pest, another is its ability to disperse over large areas to infest rice crops (Fig. 1.2). The key to its successful long distance migratory nature is that *N. lugens* is a wing dimorphic pest, displaying both macropterous and brachypterous adults.

Wing dimorphism is a feature that is seen across many insect species (Roff and Fairbairn, 1991). The frequencies of brachypterous/macropterous adults within a population depends on the population density and nutrient availability. A higher population density within the nymphal stage will lead to a comparatively higher level of macropterous adults (Mochida and Okada, 1979). The macropterous adults become abundant when *N. lugens* depletes its current food source, and therefore needs to migrate to fresh rice crops to survive. This long distance migratory potential enables *N. lugens* to reach rice in temperate regions where it cannot overwinter and so it also infest crops in Japan, Korea, northern India and China (Perfect and Cook, 1994). Once migrated the subsequent generations of adult *N. lugens* will be predominantly brachypterous, due to their enhanced rates of fecundity compared with macropterous adults (Denno, 1994). This enables the pest to rapidly reach high levels of infestation in the newly invaded areas.

A combination of factors explains the evolution of *N. lugens* from occasional pest to an endemic pest that consistently threatens rice production. The modern practice of growing rice all year round in a monoculture means there is always an ample supply of habitat for *N. lugens* to thrive on, and is a significant contributing factor to this pest's status (Bottrell and Schoenly, 2012). Bottrell *et al* also argue that the extensive use of nitrogen fertiliser is another contributing factor, since it increases *N. lugens*'s reproductive potential (Dyck and Thomas, 1979), allowing it to cause more damage to rice crops. The heavy use of chemical insecticides over a long period, and the consequent depletion of natural enemies is the other key factor in this pest's emergence as the greatest current threat to rice production throughout Asia.



Source: www.cabi.org/isc/datasheet/36301

Fig. 1.2 A map demonstrating the distribution of *N. lugens*. Blue dots represent widespread existance of *N. lugens*, with red dots representing reports of *N. lugens* without further details

1.4 Integrated pest management of *N. lugens*

The threat of *N. lugens* to rice production in Asia led to a conference being organised by the International Rice Research Institute (IRRI, 1979) to analyse the main contributing factors responsible for *N. lugens* outbreaks and to formulate a plan to better control this pest. This conference decided that an integrated pest management (IPM) programme must be implemented to contain the damage that *N. lugens* could inflict upon rice yields (Bottrell and Schoenly, 2012). An IPM strategy aims to utilise multiple methods of control including biological, chemical, cultural and physical, instead of over reliance on a single method.

Tropical Asia adopted such a scheme in 1980 for management of rice pests (Gallagher, Ooi and Kenmore, 2009). This scheme highlighted the importance of preserving populations of natural enemies and a reduction in application of chemical

compounds. There was a substantive drive to provide training to farmers to reduce pesticide use, whilst maintaining rice yield (Matteson, 2000).

Current guidelines for controlling planthoppers are provided by the Rice Knowledge Bank (IRRI, 2017). These broadly follow the same pattern as seen in the original IPM strategies, which work to prevent outbreaks from occurring in the first place, and if there is an outbreak to use mechanical/biological methods first.

There are many natural enemies (244) of *N. lugens,* including species classed as predators and others as parasitoids that can act as biological control (Gurr *et al.*, 2011). The green mirid bug, *Cyrtorhinus lividipennis*, is an important predator of eggs and early instars (Lakshmi, Krishnaiah and Katti, 2010). Other predators include the linyphiid, *Atypena formosana* and the lycosid *Pardosa pseudoannulata* (Sigsgaard, 2007). Parasitoids belonging to Hymenoptera, including the families *Dryinidae* and *Mymaridae* can also disrupt *N. lugens* populations (Gurr *et al.*, 2011). Effectively maintaining levels of natural enemies in the field, could help contribute to keeping *N. lugens* mediated damage below economic thresholds.

The Rice Knowledge Bank advice regarding chemical control is that it should only be applied if the following criteria apply: more than one *N. lugens* per stem, *N. lugens* outnumber natural enemies and that seedbed flooding is not a viable option.

1.5 Chemical control of insect pests

Throughout our recent history the predominant method of insect pest control has been chemical compounds. The use of such compounds is widespread and has been used for centuries to negate the effect of insects upon crops, livestock and humans. Nowadays this equates to the mass production and deployment of synthetic

chemicals. The first such synthetic chemical was the organochlorine insecticide DDT, which was widely used to control agricultural pests. DDT was also the key chemical used to control mosquitos that were the vector for the *Plasmodium sp* parasite responsible for malaria in humans. The majority of compounds used for chemical control are neuroactive insecticides that result in a rapid kill (Casida and Durkin, 2013). Since these target the nervous system of insects there are only a few suitable targets available. The four major nerve targets are acetylcholinesterase (AChE), nicotinic acetylcholine receptor (nAChR), voltage-gated sodium channel (VGSC) and the GABA-gated chloride channel (Casida and Durkin, 2013). The chemical classes that have become the most successful (in terms of sales) are the pyrethroids, organophosphates and neonicotinoids. These chemical classes each target a different specific nerve target within the insect nervous system.

1.6 Insecticides used to treat *N. lugens*

Heinrichs gives a summary of the early compounds used against *N. lugens*, from 1670 to the 1970s. The use of whale oil became commonplace in Japan by 1840, after its discovery in 1670 as an effective chemical control agent, and was later replaced in 1897 by kerosene (Heinrichs, 1979). Subsequent compounds (what we now consider as modern insecticides) included DDT dust and BHC (benzene hexachloride), with BHC credited as the first insecticide used for *N. lugens* control in Japan (Nagata, 1984). However, it wasn't until the 1950s that one of the first major insecticide classes, the organophosphates, was introduced for control of various insect rice pests. This included compounds such as malathion, parathion and diazinon. The 1960s saw the introduction of the carbamate insecticides, which replaced organophosphates within Japan (Heinrichs, 1979; Nagata, 1984). The next

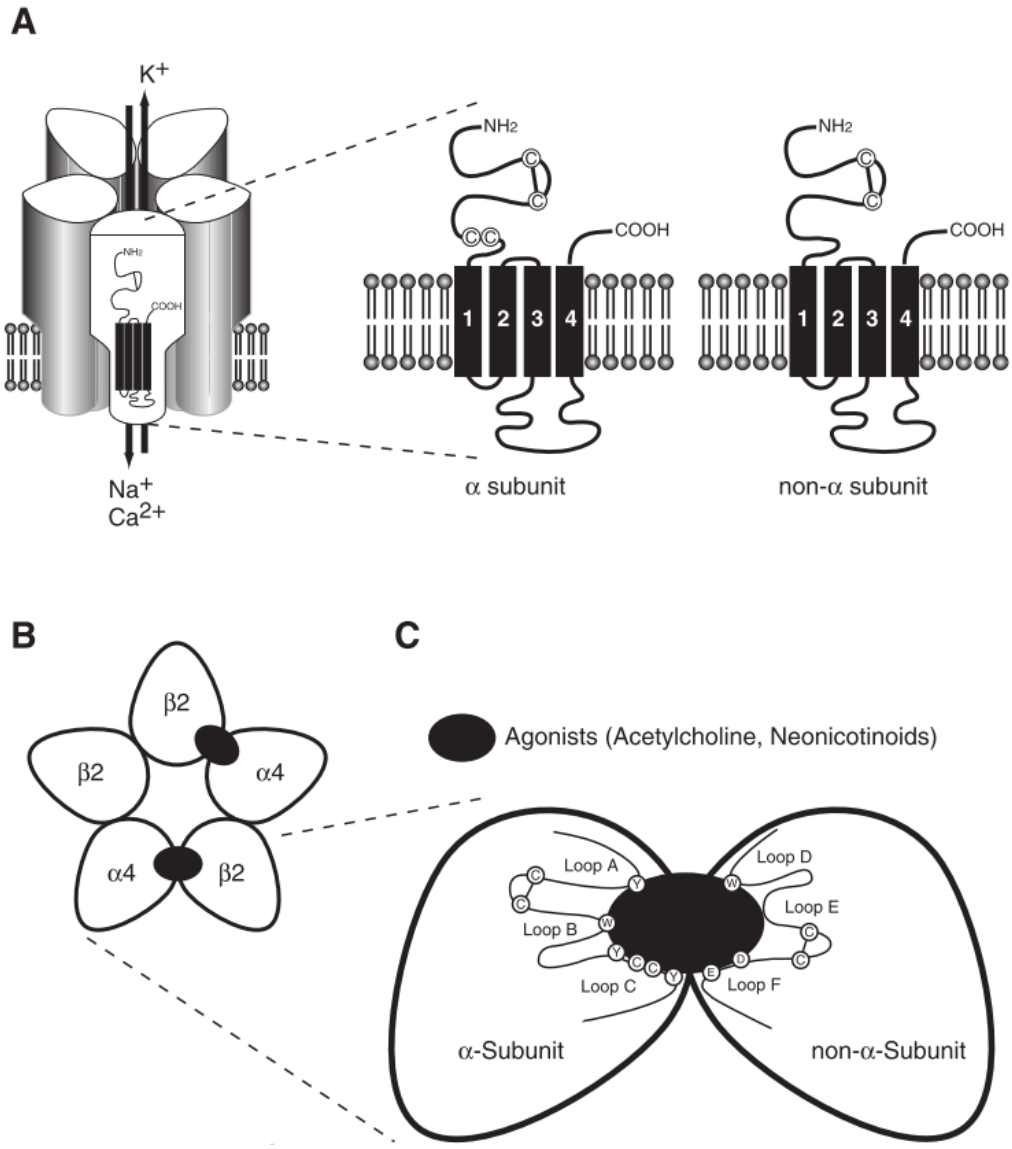
major class of insecticide developed and employed in planthopper control were the synthetic pyrethroids (Noda, 2009), which were eventually superseded in the 1990s, when the neonicotinoid class of insecticides became commercially available and were widely adopted to control *N. lugens* within rice (Matsumura *et al.*, 2008). Following on from the neonicotinoids there was a shift to the use of phenylpyrazoles (ethiprole and fipronil), which steadily rose to prominence as the primary insecticides used for *N. lugens* control (Bayer CropScience, 2007). Recently, in the last decade, an anti-feedant (pymetrozine) has been included in the arsenal of chemical compounds used against *N. lugens* (He *et al.*, 2011). The diverse range of chemical compounds introduced over the past five decades for *N. lugens* control have, unfortunately, all had their effectiveness compromised at some stage by the evolution of insecticide resistance in *N. lugens*.

Currently the neonicotinoids (e.g. imidacloprid), phenylpyrazoles (ethiprole, fipronil) and anti-feedants (pymetrozine) are the key classes of insecticides being used for chemical control of *N. lugens*.

1.7 Neonicotinoids

The neonicotinoid class of insecticides belongs to group 4A in the Insecticide Resistance Action Committee's Mode of Action (MoA) classification scheme, and act as agonists of the postsynaptic nicotinic acetylcholine receptor (nAChR) in the central nervous system of insects (Nauen *et al.*, 2001; Jeschke and Nauen, 2008). The nAChRs are pentameric membrane proteins (Fig. 1.3A) that cause rapid membrane depolarisation at synapses, and are mediated by the excitatory neurotransmitter acetylcholine (ACh) (Fig. 1.3B) (Matsuda *et al.*, 2009). Within this pentameric

structure there are six separate loops (A-F) that form the ACh binding site (Fig. 1.3C) (Matsuda *et al.*, 2005). Located within these loops are the key amino acid residues that allow docking of the endogenous agonist (ACh) and other agonists. Stimulation of the nAChR is terminated by acetylcholinesterase (AChE) which catalyses the breakdown of ACh into acetate and choline, so removing the agonist from the nAChR channel. However, when a neonicotinoid insecticide binds, the insecticide cannot be removed by AChE and so remains permanently bound to nAChR. This causes overstimulation of the nervous system which in turn leads to paralysis of the insect and then death. Extensive studies have been conducted demonstrating that the binding site of the neonicotinoids is the insect nAChR channel (Buckingham *et al.*, 1997). Since then a wide range of models have been developed that predict binding mechanisms of neonicotinoids to insect nAChR (Jeschke and Nauen, 2008; Jeschke, Nauen and Beck, 2013).



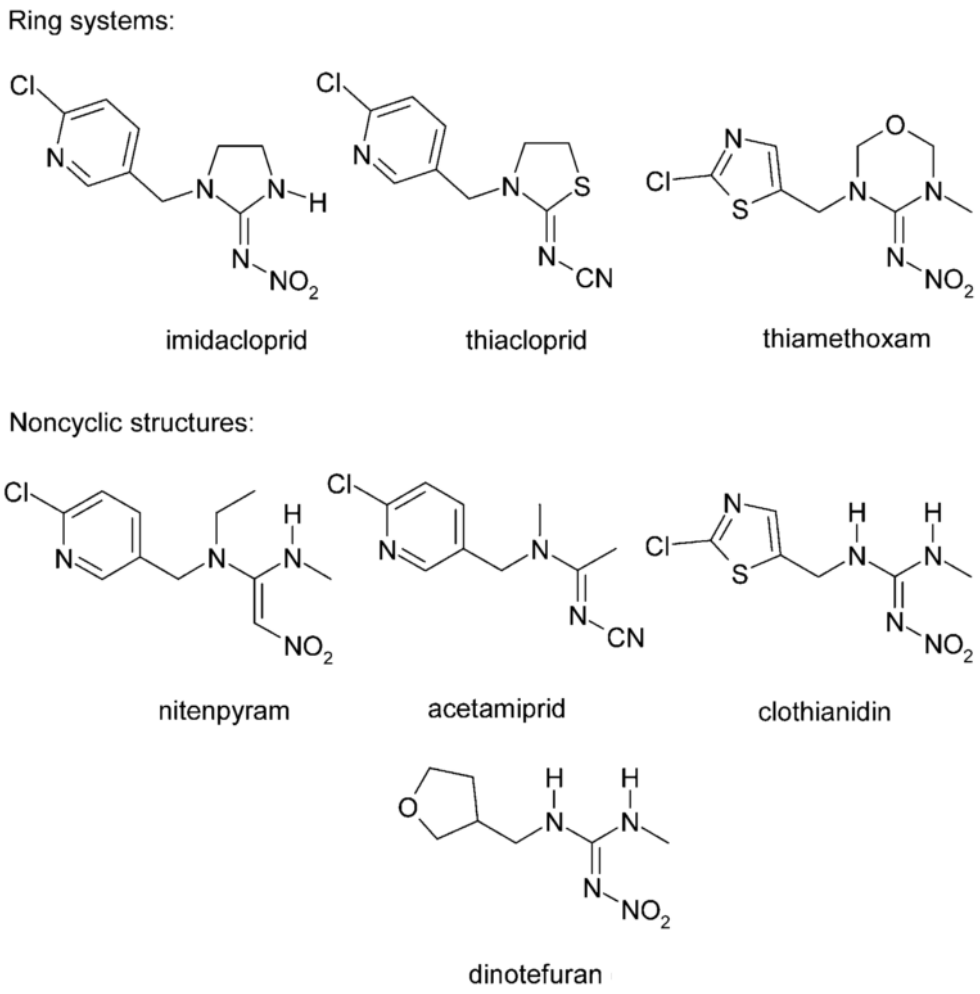
Source: Matsuda *et al.*, 2005

Fig. 1.3 A schematic display of nAChR. A) Side view. B) Top view. C) The ligand binding site within nAChR.

In the 1970s a compound, nithiazine, discovered by scientists working for Shell, displayed promising insecticidal activity. This compound is now regarded as the fore-runner of the modern day neonicotinoids (Soloway *et al.*, 1979). However, despite having low mammalian toxicity and effective systemic action within plants (Tomizawa and Casida, 2003) nithiazine was not viable as a widespread chemical control compound as it lacked photostability, and was therefore rapidly degraded

under field conditions (Soloway *et al.*, 1979). Nevertheless, studies on this compound eventually led to the development of the photostable derivative imidacloprid (Kagabu, 1997).

There are currently seven commercially available neonicotinoid compounds on the market. These can be subdivided into two categories: cyclic compounds (that include imidacloprid, thiacloprid, thimethoxam) and open chain compounds (nitenpyram, acetamiprid, clothianidin, dinotefuran) (Fig 1.4). These compounds command a large share of worldwide insecticide sales (28.5% of US$ 12.75 billion in 2011) (Jeschke, Nauen and Beck, 2013). Imidacloprid was the first compound commercially available, developed and patented in the late 1980s by Bayer CropScience. It quickly became one of the most successful compounds ever utilised for insect pest control. The primary reason for the popularity and widespread use of the neonicotinoid class is their specific activity against insects, and low mammalian toxicity (Tomizawa and Casida, 2003). The neonicotinoid compounds were also seen as more environmentally benign than older, more toxic compounds such as the pyrethroids, carbamates and organophosphates (Jeschke, Nauen and Beck, 2013). A third reason for the rapid adoption of the neonicotinoids was the fact that this class was not affected by resistance that had developed to older (preceding) insecticide classes (Denholm *et al.*, 2002).



Source: Jeschke, Nauen and Beck, 2013

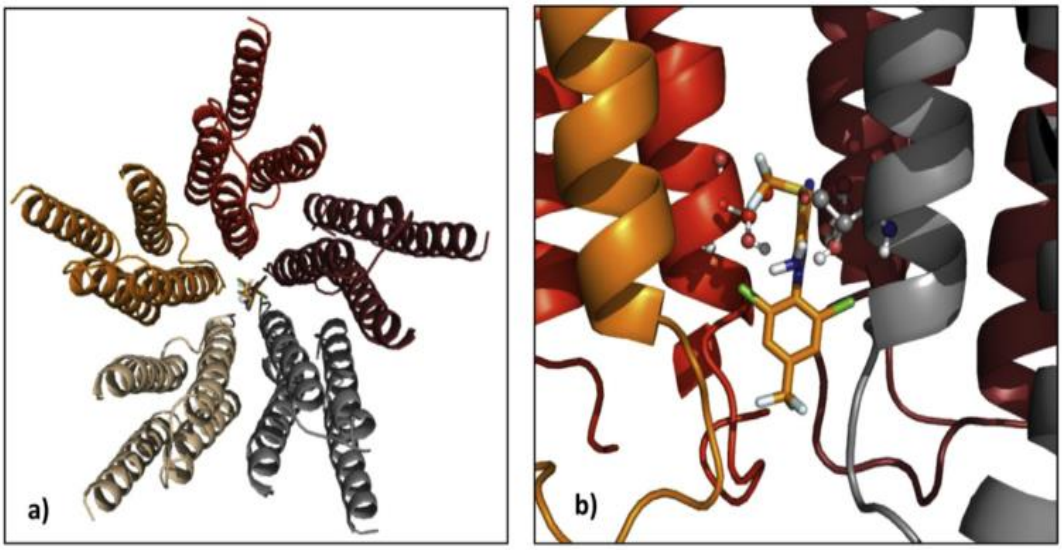
Fig. 1.4 Chemical structures of the seven commercially available neonicotinoids.

1.8 Phenylpyrazoles

The phenylpyrazole (fiprole) family of insecticides consists of three compounds; fipronil, ethiprole and pyriprole. They belong to group 2B of the IRAC MoA classification scheme, and their mode of action is as non-competitive blockers of the GABA-gated chloride channel (Cole, Nicholson and Casida, 1993; Bloomquist, 2001). The binding of the phenylpyrazoles inhibits the influx of chloride ions in nerve cells, causing hyperexcitation of the nervous system (Nakao *et al.*, 2013). The channel is encoded for by the *Resistance to dieldrin* (*Rdl*) gene, and was originally cloned from

Drosophilia melanogaster (ffrench-Constant *et al.*, 1991). This channel has also been the target of older insecticidal chemistries, primarily the cyclodiene hydrochlorines.

Insect GABA receptors differ significantly from vertebrate $GABA_A$ receptors. Vertebrate $GABA_A$ receptors are formed from different combinations of α, β or ϒ subunits, creating a complex pentamer structure (ffrench-Constant *et al.*, 2016). Insect GABA receptors are homo-oligomeric channels composed of five RDL subunits (Buckingham *et al.*, 2005). Each of these subunits has a large extracellular agonist-binding N-terminal domain (Nakao *et al.*, 2013). The GABA gated chloride channel also contains four transmembrane regions, designated M1-M4 (Whiting, 2003). It has been predicted that non-competitive antagonists (NCA) of this channel bind to the M2 transmembrane region. The crucial amino acids for binding of the NCA compounds are predicted to be: A301, T305 and L309 (all within M2) (Hisano *et al.*, 2007; Casida and Tomizawa, 2008). Molecular modelling studies have been conducted *in silico* to further analyse the docking of phenylpyrazoles in the RDL channel (Remnant *et al.*, 2014). Remnant et al. found that fipronil bound best to the lower part of the channel pore (Fig. 1.5A), via interaction with A301, L302 and T305, which were previously predicted to be the key amino acids involved in phenylpyrazole binding. Furthermore, their modelling studies conclude that the $C(O)CF_3$ group of phenylpyrazoles can form hydrogen bonds with T305 in three subunits of RDL (Fig. 1.5B) (Remnant *et al.*, 2014).



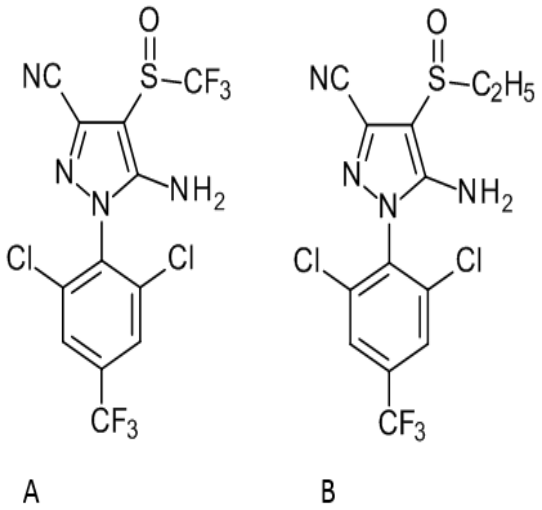
Source: Remnant *et al.*, 2014

Fig. 1.5 Fipronil bound to the transmembrane region of RDL A) A view looking down through the GABA gated chloride channel into the cytoplasm. B) A side view displaying the binding of fipronil to T305 of multiple subunits.

The forerunner phenylpyrazole was fipronil, developed by Syngenta and released commercially in 1993. (Remnant *et al.*, 2014). Fipronil demonstrates efficacy against a wide range of pests, both in a veterinary and agricultural setting. Ethiprole (developed by Bayer CropScience (Bayer CropScience, 2007)), and pyriprole (introduced by Novartis in 2000) are effective against a narrower range of pests. Pyriprole is currently specifically licenced as a veterinary product effective against various tick species and fleas (Barnett *et al.*, 2008).

Both fipronil and ethiprole have been extensively used as a control agent against *N. lugens* across Asia (Garrood *et al.*, 2016). Caboni et al. completed an in depth analysis comparing ethiprole and fipronil, studying their photochemistry, metabolism and GABAergic action (Caboni, Sammelson and Casida, 2003). In contrast to the neonicotinoid family, which has a diverse range of structures, the compounds

in the phenylpyrazole family are highly similar in structure. The only difference between ethiprole and fipronil is an ethylsulfinyl substituent replacing the trifluoromethylsulfinyl moiety (Fig. 1.6) (Caboni, Sammelson and Casida, 2003). Due to this minor structural difference ethiprole is considerably less lipophilic than fipronil. Sunlight exposure causes ethiprole to undergo oxidation, reduction and desethylsulfinylation, whilst for fipronil the major reaction is desulfinylation (Caboni, Sammelson and Casida, 2003). However, despite these differences, the study concluded that both compounds are very similar in insecticidal potency.



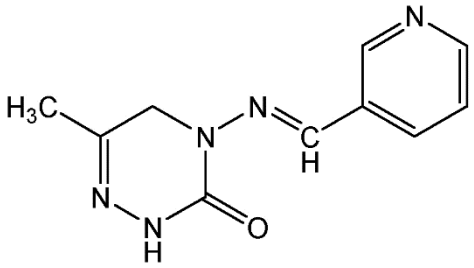
Source: Garrood *et al.*, 2017

Fig. 1.6 Chemical structure of A) Fipronil. B) Ethiprole.

1.9 Anti-feedant (pymetrozine)

One of the few insecticides registered for *N. lugens* control that has not yet been affected by high levels of resistance is the neuroactive pyridine azomethine derivative, pymetrozine (Fig. 1.7). It belongs to group 9 of the IRAC MoA classification scheme. Known as an anti-feedant (Harrewijn and Kayser, 1997), it does not cause

rapid kill but rather impacts upon feeding and reproduction capability (Tsujimoto *et al.*, 2015). Pymetrozine increases the duration of non-probing periods and inhibits phloem ingestion (He *et al.*, 2011). Studies performed on locusts revealed that pymetrozine affects insect chordotonal mechanoreceptors (Ausborn *et al.*, 2005), but until recently the precise mechanism by which this insecticide acts on sucking insects had not been fully characterised. However, studies have now concluded that pymetrozine modifies a transient receptor potential (TRP) ion channel complex, found only in insect stretch receptor cells (Nesterov *et al.*, 2015).



Source: Chem Service inc, PA, USA

Fig. 1.7 Chemical structure of pymetrozine.

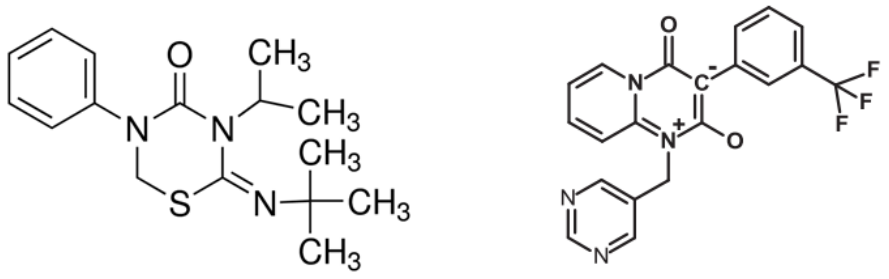
Since, due to its mode of action, this insecticide is much slower acting, it requires a different method of bioassay assessment for possible resistance. The current IRAC Susceptibility Test Method (method number 5) for *N. lugens* (IRAC, 2012), is a rather inefficient bioassay for anti-feedant insecticides, taking a long time to carry out and often leading to ambiguity in interpretation of the results. A more effective method has been demonstrated for anti-feedants using fluorescent dyes, e.g. for *Bemisia tabaci* (Cameron *et al.*, 2013). For *N. lugens* a method was recently developed that combined topical application and measurement of offspring number (Tsujimoto *et al.*, 2015), which produces a median effective dose (ED_{50}) value.

Tsujimoto et al. argue that the benefit of this is that it provides a value of 'pesticide directly applied to insects per gram of body weight'.

1.10 Other insecticides

There are two other insecticides that are relevant to brown planthopper control. The first is buprofezin (Fig. 1.8A), an insect growth regulator that was registered for use in the 1980s. Buprofezin is a chitin synthesis inhibitor developed by Nihon-Nohyaku (Wang *et al.*, 2008), which belongs to group 16 of the IRAC MoA classification scheme. It is renowned for its effectiveness against Homopteran insect pests, with low environmental and mammalian toxicity (Asai *et al.*, 1985; Nagata, 1986). It does not cause a rapid kill but rather disrupts chitin deposition during the molting process and prevents the insects from shedding the exuviae, causing death at the nymphal stage (Izawa *et al.*, 1985; Uchida, Asai and Sugimoto, 1985). It is also affects fecundity and egg hatching, causing a reduction in population growth (Uchida, Asai and Sugimoto, 1985; Ishaaya, Mendelson and Melamed-Madjar, 1988; Wang *et al.*, 2008). It was often used for *N. lugens* control in China until it was replaced by imidacloprid.

The second insecticide is triflumezopyrim (Fig. 1.8B). This compound has only recently been developed and marketed by DuPont Crop Protection, and has been assigned to a novel class of insecticides, the mesoionics (Group 4E of IRAC MoA classification scheme). Cordova et al. demonstrated that the mesoionics inhibit the orthosteric binding site of nAChR, in contrast to neonicotinoids (also group 4 insecticides) which activate the nAChRs (Cordova *et al.*, 2016).



A B

Source: A) Sigma-aldrich. B) Cordova *et al.*, 2016

Fig. 1.8 Chemical structures of A) Buprofezin. B) Triflumezopyrim.

1.11 Insecticide resistance

The over-reliance on and the repeated use of insecticides of the same chemical class without consideration for the need for MoA (IRAC group 1-29) rotation has had the inevitable consequence of resistance arising within pest populations. IRAC's given definition of resistance (IRAC, 2016) is 'a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species'.

The earliest scientific study highlighting resistance was conducted by Melander, who noted that applications of the inorganic insecticide, sulfur-lime, were not as effective as previously against the San José scale and brown mites (Melander, 1914). The following decades saw further reports of incidents of resistance, in red scale against hydrogen cyanide (Quayle, 1938) and the codling moth against lead arsenate (Hough, 1928). However, the onset of resistance to DDT, an organo-chlorine insecticide, in house-flies (Wiesmann, 1947; Harrison, 1950) demonstrated that resistance could likely arise against any compound used as a control agent. This has

ultimately proved to be the case for every new class of insecticide released to combat insect pests.

The phenomenon of insecticide resistance can be viewed as the best example of natural selection available, as it is a relatively rapid phenomenon, sometimes occurring within a few generations. How quickly resistance develops depends on a combination of factors involving the chemical used for control and the biology of the pest species. On the pest side the generation time, ability to migrate and host range, as well as the number of nearby susceptible populations are key factors. These combine with the specificity of the chemical used, when it is applied, how often and also the concentration used (IRAC, 2013) as key factors that determine how quickly resistance is selected for.

The nature of the mechanisms responsible for insecticide resistance have been extensively studied, leading to a complex and diverse picture of the genetic causes. The genetics can be monogenic or polygenic in nature (ffrench-Constant, 2013), though primarily monogenic mechanisms have been found. There are two major ways whereby resistance has arisen; 1) target site alteration, called target-site resistance, and 2) enhanced activity of detoxification enzymes, termed metabolic resistance. Other less common mechanisms are penetration resistance and behavioural resistance. All four of these mechanisms will be discussed briefly later.

Insects can utilise many of these mechanisms within a single individual, giving them resistance to a range of compounds. The clearest example of this is seen in the history of insecticide resistance in *Myzus persicae*, the peach potato aphid (Bass *et al.*, 2014). No less than seven mechanisms have been attributed to causing resistance

in this pest, starting with overexpression of E4/FE4 esterase against organophosphates (Devonshire and Moores, 1982). A mutation, S431F, in AChE causes resistance to dimethyl carbamates (Nabeshima *et al.*, 2003), whilst multiple mutations (L1014F, M918T and M918L) in VGSC have been attributed to resistance against pyrethroids (Williamson *et al.*, 1996; Eleftherianos *et al.*, 2008; Fontaine *et al.*, 2011). A further target-site mutation, A301G, in the GABA gated chloride channel implements resistance against cyclodienes (Anthony *et al.*, 1998). Overexpression of a P450 enzyme, CYP6CY3, causes resistance to nicotine and cross resistance to neonicotinoids (Puinean, Denholm, *et al.*, 2010; Bass *et al.*, 2013). Resistance to neonicotinoids may also be mediated by reduced penetration of neonicotinoids (Puinean, Denholm, *et al.*, 2010; Bass *et al.*, 2013), as well as a target-site mutation, R81T, in nAChR (Bass *et al.*, 2011). Consequently, some *M. persicae* populations can at any one time be resistant to a range of different chemical compounds or have more than one mechanism that makes them resistant to an individual chemistry. This versatility against insecticides, displayed by *M. persicae*, gives a snapshot of the different mechanisms that can evolve in an insect pest against xenobiotics. For the brown planthopper there have also been a range of mechanisms shown to provide insecticide resistance. Gene amplification, increased expression and activity of an esterase (Nl-EST1) caused resistance to organophosphates and carbamates, whilst a GST, (nlgst1-1) was shown to confer resistance to pyrethroids (Vontas *et al.*, 2002). Resistance to neonicotinoids has been linked to overexpression of P450s (Bass, Carvalho, *et al.*, 2011), and mutations in the *Rdl* gene have been linked with resistance to phenylpyrazoles (Y Zhang *et al.*, 2016; Garrood *et al.*, 2017)

1.12 Metabolic resistance

There are three enzyme families that are most commonly associated with metabolic resistance; cytochrome P450 monooxygenases (P450s), glutathione S-transferases (GSTs) and the carboxylesterases (CCEs). The primary way these enzymes cause metabolic resistance is through overexpression in response to the presence of a xenobiotic compound (Small and Hemingway, 2000; Vontas, Small and Hemingway, 2000, 2001; Vontas *et al.*, 2002; Bass *et al.*, 2011). This increased abundance of enzyme is then able to disrupt the xenobiotic and prevent it from reaching its intended target site. The mechanisms underlying this change in expression are varied, with gene amplification the most regularly reported (Devonshire and Field, 1991; Feyereisen, 1995; Bass and Field, 2011). Another mechanism is mediated by regulatory cis- and trans- elements that influence levels of gene expression (Grant and Hammock, 1992; Feyereisen, 1995). Other than enhanced expression of an enzyme, there can also be mutations present in the enzymes coding sequence that result in an increased metabolism of insecticide (Claudianos, Russell and Oakeshott, 1999). An overview of each of the three enzyme families, and respective roles in insecticide resistance is given below.

The P450s are recorded as one of the largest and oldest gene super-families, containing a very diverse range of enzymes (Feyereisen, 1999). *D. melanogaster,* the first insect genome sequenced, was found to have 85 CYP (cytochrome P450) genes (Tijet, Helvig and Feyereisen, 2001; Feyereisen, 2006). Feyereisen discusses the differences in the numbers of CYP genes found across insect orders. For example, *Apis mellifera* (hymenoptera) has only 46 CYP genes, in contrast *Tribolium castaneum* (coleoptera) has 143 CYP genes (Feyereisen, 2006). *N. lugens* has a comparatively low

number of CYP genes (67) (Xue *et al.*, 2014). The CYP genes within each insect order are separated into four distinct clades; CYP2, CYP3, CYP4 and mitochondrial (Feyereisen, 2006).

Examples of P450 mediated resistance are now widespread throughout the literature, including CYP6G1 against imidacloprid in *D. melanogaster* (Daborn, J. L. Yen, *et al.*, 2002), CYP6CY3 against neonicotinoids in *M. persicae* (Puinean *et al.*, 2010) and CYP6CM1 against imidacloprid in B and Q biotypes of *B. tabaci* (Karunker *et al.*, 2008). These mechanisms all fall into the previously discussed category of overexpression of P450 enzyme. However, more research has now been conducted on coding sequence variation of CYP genes, showing resistance caused by P450s can be more varied than just overexpression. A study into pyrethroid resistance in *Anopheles funestus*, a key vector of malaria in Africa, indicated that allelic variation in P450s was responsible for this resistance (Ibrahim *et al.*, 2015). Analysis of two genes, *CYP6P9a* and *CYP6P9b*, revealed that different alleles were associated with the resistant *A. funestus* populations compared to the susceptible, and that these genes were undergoing directional selection. Qualitative changes in CYP6P9b (V109I, D335E and N384S) were identified as being crucial in causing pyrethroid resistance through enhancing the metabolic activity of the P450 enzyme (Ibrahim *et al.*, 2015).

GSTs have been demonstrated as a mechanism of resistance against OPs, organochlorines and pyrethroids. GSTs are phase II metabolic enzymes that conjugate reduced glutathione (GSH) to the electrophilic centres of lipophilic compounds (Li, Schuler and Berenbaum, 2007). As with the previously discussed P450 mediated resistance, GST mediated resistance is primarily caused by elevated

levels of enzyme activity. A DDT resistant strain of *D. melanogaster* (PSU-R) displayed heightened levels of a Delta GST, DmGSTD1 (Tang and Tu, 1994), whilst an OP resistant *M. domestica* strain had increased levels of *MdGSTD3* transcripts (Syvanen, Zhou and Wang, 1994). Various mosquito species have also displayed elevated GST levels conferring resistance to DDT, for example AgGSTE2 in *A. gambiae* (Ortelli *et al.*, 2003) and AaGSTE2-2 in *A. aegypti* (Lumjuan *et al.*, 2005). A mutation (L119F) in *GSTe2* was also seen in *A. funestus* that was demonstrated to underlie resistance to DDT and pyrethroids (Riveron *et al.*, 2014). An enlarged DDT-binding cavity in the enzyme with the L119F mutation was behind the resistance to DDT.

The carboxylesterase enzymes hydrolyse ester bonds via the addition of water (Wheelock, Shan and Ottea, 2005). Xenobiotic compounds that are esters (e.g. carbamates, OPs and pyrethroids) have been particularly prone to CCE mediated resistance. As previously discussed, resistance is generally caused via elevated enzyme levels and/or a change in the coding sequence of the enzyme. Extensive study of the Australian sheep blowfly, *Lucilia cuprina*, indicated that OP resistance was conferred by a mutated carboxylesterase. A G137D mutation in the esterase isozyme, E3, caused the enzyme to lose carboxylesterase activity and gain the ability to hydrolyse an OP, diazinon (Newcomb *et al.*, 1997). A further mutation in E3, W251L, demonstrates hydrolysis activity against the OP malathion (Campbell *et al.*, 1998). The overview of insecticide resistance in *M. persicae,* provided earlier in this introduction, mentioned the role of esterases (E4 and FE4) in insecticide resistance. In this case the esterase genes were greatly amplified (up to 80 copies) and the level of amplification correlates broadly with the increasing levels of resistance seen in the aphids clones (R_1, R_2 and R_3) (Field *et al.*, 1999; Bass *et al.*, 2014). The amount of

esterase produced can be substantial, up to 3% of total protein in the most resistant clones (R_3), which can have a detrimental fitness cost in the absence of insecticide (Bass *et al.*, 2014). This is also an example of an esterase that sequesters, rather than hydrolyses the xenobiotic. Esterase gene amplification also occurs in the mosquito's *Culex tritaeniorhynchus* and *C. quinquefasciatus*, with *CtrESTβ1* (orthologous to *C. quinquefasciatus* ESTβs) showing elevated levels of activity due to gene amplification (Karunaratne *et al.*, 1998). One of the co-amplified genes, *ESTβ2*, in *C. quinquefasciatus* has also shown highly active promoter activity in the resistant Pel-RR strain (using a luciferase expression system) when compared with the wild-type promoter (Hawkes and Hemingway, 2002; Li, Schuler and Berenbaum, 2007).

A further gene family involved in metabolic resistance is the ABC gene family. These are part of phase III metabolism of detoxification, after phase I (P450s and CCEs) and phase II (GSTs) (Dermauw and Van Leeuwen, 2014). The ABC gene family encode for ABC (ATP binding cassette) transporters that are responsible for removal of metabolites of earlier phases of detoxification. There has been limited evidence provided so far for a role of this gene family in neonicotinoid or phenylpyrazoles resistance (Dermauw and Van Leeuwen, 2014). Dermauw et al. provide a summary of the possible cases of ABC transporters involvement in neonicotinoid and fiproles resistance. *Apis mellifera* saw increased mortality to imidacloprid, acetamiprid and thiacloprid, when verapamil (used as a synergist for ABC transporters) was applied (Hawthorne and Dively, 2011). However, no specific ABC subfamily has been linked with this, and so more evidence would be needed before calling ABC transporters a significant mechanism in resistance here. Implication of ABC transporters in resistance through upregulation in microarray/RNA-seq studies are also seen.

Upregulation of an ABCG transporter gene in *B. tabaci* thiamethoxam resistant population has been identified (Yang *et al.*, 2013), whilst multiple ABC transporters are upregulated in resistant strains of *L. striatellus* (Sun *et al.*, 2017). For a fipronil resistant strain of *P. xylostella* multiple ABC transporters were seen as upregulated (You *et al.*, 2013).

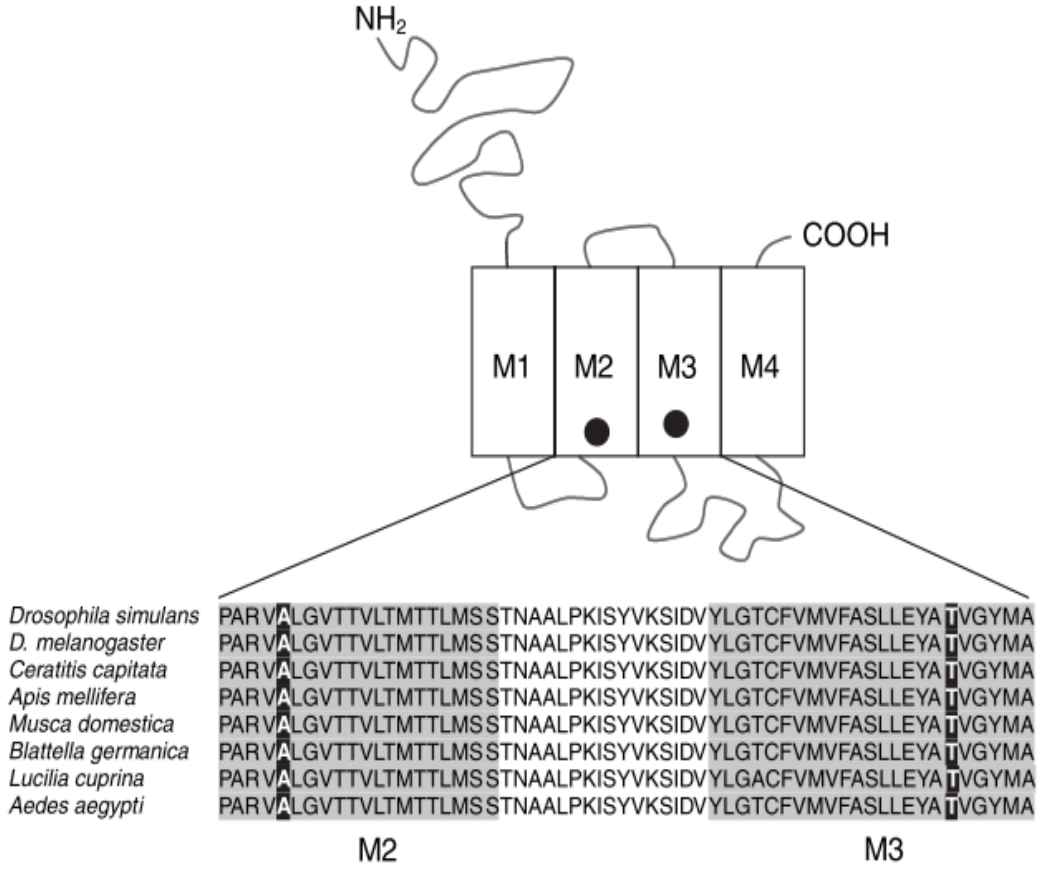
1.13 Target-site resistance

Target-site resistance occurs when an alteration in the target protein prevents the insecticide from binding/acting in the way it should. Most cases involving target-site resistance are due to a mutation in the gene encoding the insecticide target site (SNP resulting in an amino acid substitution) leading to a conformational change in the binding site. The proteins that are most well documented for developing this form of resistance are the transmembrane proteins and enzymes that exist throughout the insect nervous system, since these have traditionally been the main target for most insecticide classes developed for insect pest control. This mechanism is widespread and examples exist for all the major classes of insecticide.

The first report of a structural change in an insecticide target causing resistance was a substitution of an alanine to a serine (A301S) in the GABA-gated chloride channel, linked to a high level resistance to cyclodienes in *D. melanogaster* (ffrench-Constant *et al.*, 1993). This A301S mutation had independently arisen in several insect species (Thompson, Steichen and ffrench-Constant, 1993). This mutation has also been shown to arise independently multiple times within the same species, demonstrated in the red flour beetle, *Tribolium castaneum* (Andreev *et al.*,

1999). The A301 position (Fig. 1.9) is a hotspot for mutations, with various substitutions linked to resistance (A301S/G/N) (Le Goff *et al.*, 2005; Nakao *et al.*, 2011, 2012). It has also been shown that other mutations can arise in the RDL GABA receptor in tandem with A301S, such as T350M in *D. simulans* (Fig. 1.9) (Le Goff *et al.*, 2005).

Knockdown resistance (*kdr*) confers resistance to DDT and pyrethroids. This resistance is caused by a substitution of leucine to phenylalanine at AA position 1014 (L1014F) within the VGSC, and was first described in the house fly, *Musca domestica* (Williamson *et al.*, 1996). Higher resistance levels to pyrethroids were seen when a second mutation (M918T) was present in conjunction with L1014F, and this is termed *super-kdr* (Ingles *et al.*, 1996; Williamson *et al.*, 1996). Similarly to A301S in the GABA-gated chloride channel, L1014F has been seen in a wide range of insect species (Davies *et al.*, 2007).



Source: Le Goff *et al.*, 2005

Fig. 1.9 A cartoon representation of the RDL subunit, demonstrating the location of two mutation sites, A301 and T350 (highlighted in black).

A few cases of target site alteration conferring neonicotinoid resistance have been reported. The first is a point mutation (Y151S) in two nAChR subunits of *N. lugens* that reduced the ability of imidacloprid to bind to the receptor (Liu *et al.*, 2005, 2006). This was the first demonstrated target-site resistance mechanism for neonicotinoids, but has never been found in the field. Detoxification via P450s has been the dominant mechanism of neonicotinoid resistance seen in field populations. A novel mutation (R81T) in loop D of the nAChR β1, was linked with neonicotinoid resistance in field collected *M. persicae* (Bass, Puinean, *et al.*, 2011). Downregulation of the nAChR α8 subunit was linked to imidacloprid resistance in *N. lugens* (Y. Zhang *et al.*, 2015).

1.14 Penetration resistance

Penetration resistance is far less common than either metabolic or target-site resistance. It occurs when a resistant insect is much slower to absorb the insecticide in comparison to a susceptible insect. Overexpression of several cuticular proteins in *M. periscae*, leading to reduced cuticular penetration of neonicotinoids, was seen as contributing to resistance, as well as an overexpressed P450 (Puinean, Foster, *et al.*, 2010). Another example is seen in *Plutella xylostella*, with reduced uptake of S-fenvalerate (pyrethroid) in resistant strains (Noppun, Saito and Miyata, 1989). Recently penetration resistance has been linked with pyrethroid resistance in *A. gambiae* (Bass and Jones, 2016). Resistant mosquitoes had a thicker epicuticular layer than susceptible insects, and also a larger cuticular hydrocarbon content (Balabanidou *et al.*, 2016).

1.15 Behavioural resistance

IRAC defines behavioural resistance as the ability of 'resistant insects to detect or recognise a danger and avoid the toxin' (IRAC, 2016). A classic example of this resistance is seen in the control of German cockroaches, *Blatella germanica*. There glucose based gel baits were no longer effective since the pest simply avoided eating the baits (Wang, Scharf and Bennett, 2004). The potential rise of behavioural resistance in mosquitos has been modelled and it was demonstrated that it would significantly hamper malaria control (Gatton *et al.*, 2013). Currently there is not believed to be any clear evidence for behavioural resistance in mosquitoes and has been argued that there are mosquito taxa that are behaviourally resilient rather than resistant (Govella, Chaki and Killeen, 2013). It has also been argued that the definition of behavioural resistance is too vague and new experimental approaches are

required (Zalucki and Furlong, 2017). There is a general lack of information regarding behavioural resistance currently, since it is more complex to quantify than physiological resistance and harder to screen in field populations.

1.16 Objectives

The aim of this project was to increase the understanding of how the rice pest, *N. lugens,* is able to become resistant to insecticide resistance. With a more complete picture of why chemical control has broken down for the brown planthopper, there can be a more structured approach to dealing with this major pest. It is hoped that the body of research presented in this thesis will help future research into insecticide resistance of *N. lugens* and future control programmes.

Chapter II is broad description of the general materials and methods used throughout this PhD. Chapter III monitors the levels of resistance of various *N. lugens* field strains to compounds that have become inefficient at controlling them. Chapter IV focuses on the assembly of a transcriptome and the gene expression analysis performed between a susceptible and resistant *N. lugens* populations. Chapter V focuses on the attempts to elucidate the mechanism(s) behind ethiprole/fipronil resistance by studying the gene (*Rdl*) that encodes these insecticides' target site. Chapter VI will describe the analysis of potential metabolic resistance mechanisms. This predominantly focuses on metabolic resistance to imidacloprid, but also encompasses cross-resistance mechanism studies for imidacloprid and ethiprole.

Chapter II General Materials and Methods

2.1 *Nilaparvata lugens* rearing

Live *N. lugens* strains kept at Rothamsted Research were reared in the laboratory on whole 5 to 10 weeks old rice plants (*Oryza sativa* L. ssp.) under controlled environmental conditions (26 ^{o}C, 16 h photoperiod and 70% relative humidity). Mesh cages were set up and a pot (1.1 L) of rice was added to a deep tray that was filled with water. Approximately 75-100 adults were used to populate the new cage, and fresh whole rice plants were added when instars appeared, and when older plants showed considerable signs of damage.

2.2 *N. lugens* strains

All strains were provided by Bayer CropScience (Monheim, Germany), and were collected from across South and East Asia. A summary of all the strains used in this PhD is shown below.

Table 2.1 *N. lugens* strains.

Strain	Year collected	Country of origin	Region/area
Bayer-S	1984	Japan	
NI9	August 2009	Thailand	
NI31[A]	October 2010	Taiwan	Yulin County
NI33	November 2010	Vietnam	Trà Vinh Province, Southern Vietnam
NI39	August 2011	Vietnam	Hau Giang
NI40	August 2011	Indonesia	Anjatan District, Indramayu
NI44	August 2011	Indonesia	Parnanukan District, Subang
NI45[A]	September 2011	India	Raipur, Chhattisgarth
NI52[A]	March 2012	India	Koppal District, Karnataka State
NI55	February 2012	India	East Godavari District, Andhra Pradesh
NI56[A]	April 2012	India	East Godavari District, Andhra Pradesh
NI57	September 2012	India	Kanagala District, Karnataka State
NI58[A]	September 2012	India	Mudhapur, Karnataka State
NI59[A]	September 2012	India	Sidhikerra, Karnataka State

[A]No live laboratory cultures, insects used had been preserved at -80^{o}C

2.3 *Drosophila melanogaster* rearing

Fly strains were maintained on standard food (Bloomington formulation) at 24^{o}C. Approximately 6 mL of standard food was added to standard Drosophila vials (Dutscher Scientific, Brentwood, Essex, UK). Flies were anaesthesised with CO_2 and 10-15 flies were transferred to the fresh vial. 2.5 – 3 weeks later 10-15 flies are again transferred into fresh vials.

2.4 *D. melanogaster* insecticide bioassays

3-5 day old adult females were used in insecticide bioassays to assess the susceptibility of different fly strains to technical compounds. The flies were subjected to the insecticide in a contact/feeding bioassay. Standard Drosophila vials (Dutscher Scientific, Brentwood, Essex, UK) were filled with agar solution (4ml/vial) containg 2% w/v agar (Dutscher Scientific), 1.2% w/v food grade sucrose and 0.4% v/v glacial acetic acid (Sigma Aldrich, St. Louis, MO, USA). 18 h prior to bioassay the agar vials were spread with 100 µL of insecticide solution and vortexed vigorously. For each concentration vials were prepared in triplicate for each fly strain with flies anaesthesised with CO_2 and 10 female flies added to each vial. The vials were kept upside down until all flies became active to avoid flies getting trapped in agar. The bioassay was assessed after 48 h (neonicotinoids) or 72 h (fiproles and dieldrin), dead flies as well as seriously affected flies i.e. those displaying no coordinated movement, that were unable to walk up the vial, or unable to get to their feet were cumulatively scored as ‘affected’.

2.5 Bioassay data analysis

Raw data was corrected for control mortality using Abbott's formula (Abbott, 1925). Probit analysis was performed with the the GenStat® (2014, 17th Edition, ©VSN International Ltd, Hemel Hempstead, UK) statistical software to generate LC_{50} values. Resistance ratios were calculated by dividing the LC_{50} of any given strain by the LC_{50} of the susceptible strain.

2.6 Genomic DNA extraction

DNA was extracted from single insects using 20 µL microlysis plus extraction buffer (Microzone Ltd., Haywards Heath, Sussex, UK) using the manufacturer's protocol for tough cells. Samples were placed in a thermal cycler and the following profile run: 65°C for 15 min, 96°C for 2 min, 65°C for 4 min, 96°C for 1 min, 65°C for 1 min and 96°C for 30 s. This procedure releases DNA from the insects, but does not purify it, so estimation of DNA yield via spectrophotometer was not possible. DNA extracted in this manner was suitable for use in PCR.

2.7 Total RNA extraction

Live adults were collected and flash frozen in liquid nitrogen and stored at -80°C before extraction of RNA. Total RNA was extracted using Isolate RNA mini kit (Bioline, London, UK) per the manufacturer's recommended protocol. A total of 5 insects (up to 30 mg tissue) were ground up using a pestle in 350 µL of Lysis buffer. This was centrifuged at 16 000 x g to pellet insect debris. This lysate was then filtered and 350 µL of 70% ethanol was combined with the filtrate. The RNA was then bound to the membrane of a fresh column, by centrifuging at 11 000 x g. The membrane was desalted with 350 µL of Membrane Desalting Buffer. The addition of DNase I removed any genomic DNA from the sample. The silica membrane was washed three

times and the RNA eluted into a nuclease-free 1.5 mL collection tube using 30 µL of RNase-free water. The quality and quantity of RNA was assessed via a ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). RNA was always stored at -80°C to prevent degradation.

2.8 cDNA synthesis

RNA was used for first strand cDNA synthesis using Superscript III Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA). 1-5 µg of total RNA was mixed with 0.5 µL of random primers, 0.5 µL of oligo$(dT)_{20}$, 1 µL 10 mM dNTP Mix and made up to 13 µL with sterile, distilled water. The mixture was incubated at 65°C for 5 min before being placed on ice for at least 1 min. To this 4 µL of 5X First-Strand Buffer, 1 µL 0.1 M DTT, 1 µL RNaseOUT™ Recombinant RNase Inhibitor and 1 µL of SuperScript™ III RT was added, giving a final working volume of 20 µL. Due to the inclusion of random primers, a first incubation step at 25°C for 5 min was included and followed by a further 45 min at 50°C. The reaction was inactivated by heating to 70°C for 15 min. The cDNA was stored at -20°C.

2.9 Primer design

Primers were designed to be 20-30 nucleotides in length, with a GC content between 40-60% and a salt adjusted T_m of 58-60°C. An online tool OligoCalc (**http://biotools.nubic.northwestern.edu/OligoCalc.html**) (Kibbe, 2007) was used to calculate these properties.

2.10 Standard PCR protocol

A typical PCR reaction (25 µL) contained 12.5 µL Dreamtaq (Thermo Fisher, Waltham, MA, USA) containing Taq polymerase, 2x PCR buffer and 4 mM $MgCl_2$ (2

mM final Concentration), 1 µL of forward primer (10 µM), 1 µL reverse primer (10 µM), 0.5-2 µL of gDNA/cDNA and sterile distilled water up to 25 µL. Cycling conditions were 95^{o}C for 2 min (initial denaturation) followed by 25-35 cycles of 95^{o}C for 30 s (denaturation), 5^{o}C below the lowest primer's T_m (generally 50-60^{o}C) for 30 s (annealing) and 72^{o}C for 1 min/kb (extension), with a final elongation at 72^{o}C for 5 min. A negative control was also run without the addition of gDNA/cDNA to check for possible contamination.

2.11 Agarose gel electrophoresis

Agarose gels were made by dissolving molecular biology grade agarose (Thermo Fisher, Waltham, MA, USA) in 1x TBE buffer (0.89 M Tris Borate pH 8.3 and 20 mM Na_2EDTA; National Diagnostics, Atlanta, GA, USA) to make 1-1.2% gels w/v. 0.5 µg/mL of ethidium bromide (Sigma Aldrich, St. Louis, MO, USA) was added prior to casting gels. PCR products (5 µL) were loaded into the wells of the gel and placed within an electrophoresis tank filled with 1x TBE buffer. These were run at 80V/200 mA for 1 hour. 5 µL of GeneRuler 1 kb DNA ladder (Thermo Fisher, Waltham, MA, USA) was loaded into the first well. Gels were visualised using a UV trans-illuminator (Syngene, MD, USA).

2.12 Purification of PCR products

PCR purification was performed using Wizard® SV Gel and PCR Clean-up System (Promega, Madison, WI, USA) as per the manufacturer's guidelines. For gels that contained more than one band, the desired (correct sized) band was excised using a clean razor blade and placed in a 1.5 mL microcentrifuge tube. Membrane Binding Solution was added in the ratio of 10 µL per 10 mg of gel slice and incubated at 60^{o}C until dissolved. This dissolved gel solution or prepared PCR product was then

added to a minicolumn and incubated for 1 min at room temperature before centrifuging at 16 000 x g. The column was washed twice with Membrane Wash solution before the DNA was eluted in 30 µL of nuclease-free water. Quality and Quantity were then assessed using a ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). DNA was then stored at -20^{o}C.

2.13 DNA sequencing

PCR products were sent to MWG Eurofins, Germany, for sequencing per their specifications of template concentration, dependent on product size, combined with 2 µL of 10 pmol μL^{-1} primer.

2.14 PCR Cloning

PCR products were cloned using the StrataClone cloning PCR kit (Agilent Technologies, Santa Clara, CA, USA) as per manufacturer's guidelines. A ligation reaction was assembled containing 3 µL of StrataClone cloning buffer, 2 µL of PCR product (5-50 ng), 1 µL of pSC-A-amp/kan cloning vector. and incubated at room temperature for 5 min before being placed on ice. 1 µL of the ligation reaction was added to StrataClone SoloPack competent cells that had been thawed on ice. This transformation mixture was left on ice for 20 min, before being heat-shocked at 42^{o}C for 30 s. This was then placed back on ice for 2 min before 250 µL of pre-warmed LB medium (10 g NaCl, 10 g tryptone and 5 g yeast extract per Litre) was added. This was then left to recover for at least an hour at 37^{o}C with agitation (~250 rpm). LB-Ampicillin agar plates (1 mL of 100 mg/mL ampicillin per Litre) were plated out and spread with 40 µL of 2% X-Gal. 100 µL of the recovered cells were then spread onto the agar plates and left in a 37^{o}C incubator overnight. White colonies were picked for screening whilst dark blue colonies were avoided. The colonies were collected on a

pipette tip and streaked onto a new LB-Ampicillin plate (from which material could be collected for plasmid preparation), then the tip was placed in 20 µL of sterile water. 2 µL of this water template was then used in a colony PCR reaction (same mixture as described in standard PCR protocol). Generic cloning primers, M13_F and M13_R, were used. The conditions for the cloning PCR were: 95^{o}C for 3 min followed by 30 cycles of 94^{o}C for 30 s, 60^{o}C for 30s, 72^{o}C for 1 min/Kb and a final 70^{o}C for 5 min. The samples were then assessed in the same style as for the standard PCR protocol by agarose gel electrophoresis.

2.15 Plasmid purification

Plasmids were purified using a GeneJET plasmid miniprep kit (Thermo Fisher, Waltham, MA, USA). Single colonies that had been identified as containing the correct insert were picked using a pipette tip from the colony record plate and added to 3 mL of LB ampicillin medium (1 mL of 100 mg/mL ampicillin per Litre) in a 15-mL falcon tube. This was incubated for 16 h at 37^{o}C with agitation (250 rpm) in a shaking incubator. The bacterial cells were harvested by centrifugation at 3000 x g for 10 min at room temperature. The supernatant was removed by pipetting, and the pelleted cells were re-suspended in 250 µL of resuspension solution by vortexing. The cells were transferred to a sterile 1.5 mL Eppendorf tube and mixed by inverting the tube with 250 µL of lysis solution to lyse the cells. Mixing was done by inverting the tube to avoid shearing of chromosomal DNA. This reaction was terminated within 5 min (to prevent denaturation of supercoiled plasmid DNA) by the addition of 350 µL of neutralisation solution and mixed via tube inversion. Cell debris and chromosomal DNA was pelleted by centrifuging at 16 000 x g for 5 min. The supernatant was transferred to a fresh GeneJet spin column via pipetting, with care being taken not

to disturb the precipitate. This was centrifuged at 16 000 x g for 1 min, before undergoing two wash steps with wash solution. A further centrifugation step was then necessary to remove any residual ethanol contamination of the miniprep. The spin column was placed in a new 1.5 mL tube and the DNA eluted using 50 µL of sterile distilled water. The quality and quantity of the plasmids were then assessed via spectrophotometer. The purified plasmid was then stored at -20^{o}C.

2.16 Real-time quantitative RT PCR

Primers were designed to amplify a 90-200 bp region of the gene using Primer3 program (Untergasser *et al.*, 2007). Multiple pairs of primers were designed per gene to increase the chance of finding a primer pair with an adequate efficiency, discussed later.

PCR reactions (15 µL) contained 5 µL of cDNA (2.5ng), 7.5 µL of SYBR Green JumpStart Taq Readymix (Sigma Aldrich, St. Louis, MO, USA), and 0.25 µM of forward and reverse primer. Samples were run on a Rotor-Gene Q (Qiagen, Venlo, Limburg, Netherlands) using the temperature cycling conditions of: 10 minutes at 95°C followed by 40 cycles of 95°C for 15 seconds, 57°C for 15 seconds and 72°C for 20 seconds. A final melt-curve step was included post-PCR (ramping from 72°C-95°C by 1°C every 5 seconds) to check for non-specific amplification.

To validate the primers for the experimental qRT-PCR, standard curves were created. This involved running a dilution series of cDNA at five different concentrations ranging from 50 ng/µL to 0.005 ng/µL, with three technical replicates per concentration. This allows an efficiency for the primer pair to be calculated based on the assumption that after each cycle in the program the quantity of DNA should

have precisely doubled. Therefore, primers were only chosen for assessing gene expression if they fell in the range of 0.9-1.1, with 1 representing optimal efficiency.

Each qRT-PCR experiment consisted of three independent biological replicates with two technical replicates for each. Technical replication was limited to two replicates as 1) PCR reactions were set up using a liquid handling robot (CAS 1200, Corbett Research) which provided high levels of technical reproducibility and 2) to allow us to employ a sample maximisation strategy (i.e. running as many samples as possible in the same run, to minimise technical run-to-run variation). Data were analysed according to the $\Delta\Delta C_T$ method (Pfaffl, 2001) to calculate relative gene expression values. For normalisation, two reference genes were used for each strain. For *N. lugens* samples, actin and α2-tubulin were used, whereas for *D. melanogaster* samples, Rpl32 (Ribosomal protein L32) and SDHA (succinate dehydrogenase complex flavoprotein subunit A) were used as the reference genes. The geometric means of the selected genes were then used to normalise the test samples by a previously described strategy (Vandesompele *et al.*, 2002)

2.17 SNP calling of RNA-seq reads

The RNA-seq was designed to allow a comparison of gene expression between susceptible and insecticide resistant populations. The Bayer-S strain was contrasted with two field populations (Nl33 and Nl55) and the laboratory ethiprole selected counterparts (Nl33-eth and Nl55-eth). The levels of differential expression of the transcripts assembled by Trinity were then analysed for transcripts that could potentially be involved in insecticide resistance.

Raw reads of each *N. lugens* strain were mapped to curated consensus sequences of interest to conduct single nucleotide polymorphism (SNP) analysis.

Geneious R8's (Biomatters, Auckland, New Zealand) map to reference function was used with the consensus sequence of interest as the reference. The settings applied were: 5% gaps, maximum mismatches: 10%, minimum overlap identity: 80%, index word length: 14, maximum ambiguity: 4.

2.18 Locomotor activity level monitoring (trikinetics)

Glass behaviour tubes were filled with 2-3 cm of agar by filling a beaker slowly with molten agar and placing the tubes in. The tubes were then capped, with a rubber cap, to hold the agar in place. The tubes were then allowed to cool before the agar was spread with 50 µL of insecticide solution and vortexed, then left to dry overnight. The following day 10 adult male flies, aged 2-7 days after eclosion, were added to each tube and the tubes stoppered with cotton wool. A range of five insecticide concentrations was used, with two replicates per concentration. The tubes were loaded into a DAM2 activity monitor (Trikinetics, Inc., Waltham, MA, USA), ensuring that food was not blocking the infrared beam. The loaded monitor was placed in a dark incubator and connected to the DAMSystem308 software installed on a computer. The system was set to record in 5 mins sections for all of the tubes. The experiment was left to run, maintained in darkness, for a minimum of 24 h.

2.19 *D. melanogaster* germline transformation

D. melanogaster germline transformations were performed using a modified protocol (http://carroll.molbio.wisc.edu/methods/Miscellaneous/injection.pdf). from N. Gompel This protocol was optimised for our purposes by a postdoctoral researcher, Christoph Zimmer. The *D. melanogaster* transformations were performed to allow us to functionally validate the candidate genes found in *N. lugens* using an *in vivo* experimental approach.

2 days before first injection the flies (~300) were transferred to an egg laying cage to acclimatise to conditions and incubated at 24°C. The egg laying plate which was made using grape agar premix (Flystuff, San Diego, CA, USA), was streaked with fresh yeast paste and changed twice a day.

On the day that injection took place the egg laying plates were changed 3 times in the 2 h preceding the first harvest period, to flush out old embryos from the flies. Embryos were harvested in a 30-45 min laying period and transferred to a mesh basket. After through rinsing with tap water, the embryos were transferred to a coverslip and aligned along one edge, with the posterior pole pointing to the edge. Each coverslip was packed with approximately 100 embryos lying side by side. The embryos were left to dry to attach to the coverslip, before being covered with a minimal amount of halocarbon oil mix (2.5 mL series 27 + 5 mL series 700, both Sigma-Aldrich). This was left for 8 min to penetrate between the chorion and the vitelline membrane.

The injections were done using an inverted microscope (eclipse Ti-U Nikon, Japan) equipped with a 10x/0.25 lens, 10x/22 eyepiece and fluorescence illumination. To deliver the injection mix a micromanipulation set-up of a motorised micromanipulator express microinjector (Eppendorf, Hamburg, Germany) and a FemtoJet express microinjector (Eppendorf, Hamburg, Germany) were used. Injection needles were prepared by C. Zimmer using a Stoelting APP-1 52500 All Purpose Puller (Stoelting, Dublin, Ireland) and thin wall glass capillaries (100 mm length, 1.5 mm OD, 1.12 mm ID, from World Precision Instruments, Sarasota, FL, USA) in accordance with previous research (Miller, Holtzman and Kaufman, 2002).

To inject the embryos, the filled needle was positioned so that the tip was alongside the posterior pole of the embryos. Before injecting, the injection time and pressure were calibrated so that a small droplet of liquid could be seen emerging from the needle when injecting. The embryo was gently impaled by the needle tip, taking care to remain in the first 1/5th of the embryo (where the germ cells are), and a small droplet of liquid injected. The needle was then removed in a swift motion and the injection stage moved to the next embryo to be injected. Any embryo that was overdeveloped or in the wrong orientation was over-injected to prevent further development.

After all embryos had been injected the oil was drained from the coverslip and placed into a food vial, so that the embryos were positioned just above the food. The vial was left horizontally for the first 48 h after injection at 24^{o}C, before being stored vertically at 24^{o}C. When the adult flies hatched, they were collected and crossed to the appropriate fly strain. Each male survivor from the injection would be crossed to 3 virgin females and vice versa for the surviving female

Chapter III Resistance monitoring in *N. lugens*

Some of the results presented in this chapter have been published in peer reviewed journals (reprints attached in Appendix B and C)

Garrood, W. T., Zimmer, C. T., Gorman, K. J., Nauen, R., Bass, C. and Davies, T. G. E. (2016). 'Field-evolved resistance to imidacloprid and ethiprole in populations of brown planthopper *Nilaparvata lugens* collected from across South and East Asia', *Pest Management Science,* 72**,** 140-9.

Garrood, W. T., Zimmer, C. T., Gutbrod, O., Lüke, B., Williamson, M. S., Bass, C., Nauen, R. and Davies, T. G. E. (2017) 'Influence of the RDL A301S mutation in the brown planthopper *Nilaparvata lugens* on the activity of phenylpyrazole insecticides', *Pesticide Biochemistry and Physiology*. Rothamsted Research Ltd, pp. 1–8. doi: 10.1016/j.pestbp.2017.01.007.

3.1 Introduction

The control of the brown planthopper *Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae) has for decades relied on the application of synthetic insecticides. This led to a situation in which *N. lugens* populations emerged which are resistant to several of the major insecticide classes applied, including the organophosphates, carbamates and pyrethroids (Hemingway, Karunaratne and Claridge, 1999; Nagata *et al.*, 2002). When these insecticide classes were no longer able to provide effective control in the field, the neonicotinoids became the insecticides of choice against *N. lugens*.

Since the mid-1990s, the neonicotinoid insecticide imidacloprid has been very widely used to control *N. lugens*, and this has inevitably led to the appearance of resistance to this insecticide class in the population, first witnessed in 2003 (Matsumura *et al.*, 2008). More recent monitoring has demonstrated that very high levels of resistance to imidacloprid are now present in *N. lugens* populations across Asia (Wen *et al.*, 2009; Zhang *et al.*, 2014; Garrood *et al.*, 2016). Resistance monitoring across nine regions of China revealed that imidacloprid resistance levels had increased to much higher levels by 2012 compared to 2009, with resistance ratios (RR) as high as 616.6 being reported (Zhang *et al.*, 2014). Levels of imidacloprid resistance in *N. lugens* immigrating into Japan showed near identical resistance ratios of 615.5, when comparing LD_{50} from 2012 with 1992 (Matsumura *et al.*, 2013). Matsumura *et al*. also tested two other neonicotinoids for resistance in their study, thiamethoxam and dinotefuran. For thiamethoxam there was a significant increase in LD_{50} between 2006 and 2011, from 0.27 μg g^{-1} to 4.7 μg g^{-1}, representing a 21-fold increase, whereas dinotefuran values were all below 1 μg g^{-1} between 2005-2012, but the values seen in 2012 were ~3-fold higher than in 2005. Zhang *et al*. also monitored resistance to other neonicotinoids in addition to imidacloprid, namely thiamethoxam and nitenpyram. In China, *N. lugens* resistance to thiamethoxam was at a moderate level (RR 13.9 ~ 36.7-fold) in 2011, like that seen for *N. lugens* immigrating into Japan. Testing for nitenpyram resistance in 2011 revealed that all *N. lugens* populations tested across China displayed susceptibility to nitenpyram (RR 0.96 ~ 2.4-fold), though they note that in 2012 this had increased slightly to RR 1.4 ~ 3.7-fold, and they surmise that resistance to nitenpyram could be at an early stage of development. (Zhang *et al.*, 2014).

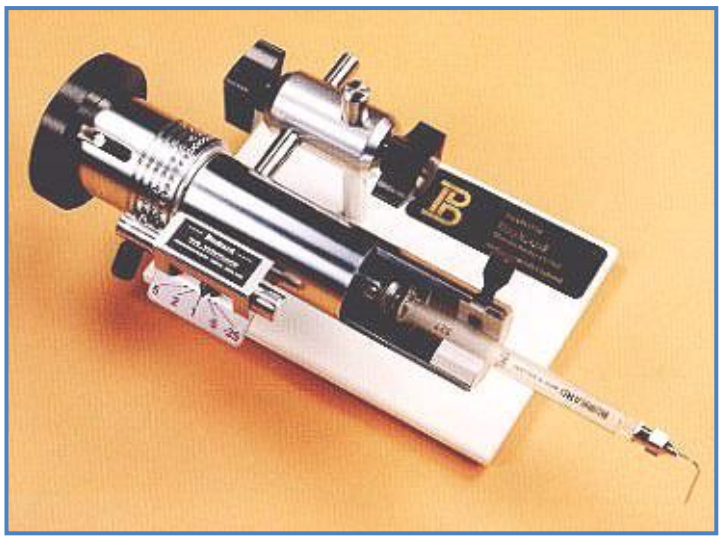
Since there was significant resistance being displayed to neonicotinoid insecticides, most notably against imidacloprid, a switch to a new class of insecticide was necessitated to maintain control of *N. lugens* populations. The insecticides of choice in this instance were the phenylpyrazoles (fiproles) insecticides, with the main products deployed being ethiprole and fipronil. Ethiprole, like imidacloprid, was first developed by Bayer CropScience (Bayer CropScience, 2007). However, the increasingly widespread use of these fiprole compounds inevitably led to resistance to this alternative chemistry emerging. By the end of the last decade *N. lugens* populations in China displayed resistance to fipronil (23.8-43.3-fold resistance) and cross-resistance to ethiprole (47.1-100.9-fold) (Wang *et al.*, 2009; Zhao *et al.*, 2011). High levels of resistance to ethiprole in *N. lugens* populations has also been seen across a number of countries in South and East Asia (Punyawattoe *et al.*, 2013; Garrood *et al.*, 2016).

To further explore the extent of resistance to neonicotinoid & fiprole insecticides in *N. lugens* populations, bioassays with the insecticides imidacloprid, ethiprole and fipronil were conducted on the *N. lugens* populations held at Rothamsted Research. Various of the field collected strains housed in the Rothamsted insectary also underwent further selection with neonicotinoid and fiprole insecticides in the laboratory to increase the resistance in these strains. Resistance bioassays were then performed to allow a comparison to the unselected (field collected) strains and to assess the extent of cross-resistance, if any, between the three compounds mentioned above.

3.2 Materials and methods

3.2.1 Topical application bioassay

Adult macropterous (long-winged) *N. lugens* were taken from age-structured populations and were less than 10 days old. Approximately 15 adults were lightly anaesthetised and dosed with the required concentration of technical grade imidacloprid (Sigma Aldrich, St. Louis, MO, USA) on the upper surface (pronotum) of the prothorax using 0.2 µL of acetone as the solvent carrier, delivered using a hand-held Burkard microapplicator (Burkard Manufacturing Co. Ltd, Rickmansworth, UK) fitted with a 1 cm^3 all-glass syringe (Fig.3.1). Control insects were dosed with 0.2 µL of acetone only. Treated individuals were placed in 50 mL specimen tubes containing untreated five-week-old rice stems (cut into 10 cm lengths) and contained using a ventilated lid. A small hole (3 mm diameter) was drilled in the base of each of the tubes, which were then stored vertically in a water bath (submerging only the base of each rice stem) in a 16 h photoperiod at 26 ^oC for 48 h (Fig. 3.2). Insect mortality at 48 h was assessed by eye; adults showing no sign of movement were scored as dead. Bioassays consisted of three replicates at each concentration. Data analysis was conducted as outlined previously (Chapter II, Section 2.5).



Source: Burkard

Fig. 3.1 Hand microapplicator (Burkard Scientific) and syringe.

3.2.2 Leaf-dip bioassay

Adults were taken from age-structured populations and were aged less than 10 days old. Rice stems (10 cm cut length) were dipped into the required concentrations of formulated insecticide for 20 s, air-dried and placed in a 50 mL specimen tube. Approximately 15 adults were aspirated directly into each tube and sealed with a ventilated lid. A small hole (3 mm diameter) was drilled in the base of each of the tubes, which were then stored vertically in a water bath (submerging only the base of each stem) in a 16 h photoperiod at 26 °C for 72 h. Insect mortality at 72 h was assessed by eye, adults showing no sign of movement were scored as dead. Bioassays consisted of 3 replicates at each concentration. Data analysis was conducted as outlined previously (Chapter II, Section 2.5).

Source: T. G. Emyr Davies

Fig. 3.2 Bioassay set-up used to assess responses of *N. lugens* to insecticide applications.

3.2.3 Laboratory selection with imidacloprid

Field strains demonstrating relatively high levels of resistance to imidacloprid were placed under further selection with imidacloprid in the laboratory. Rice plants were treated with a soil drench, whereby a solution of formulated (200 g L^{-1} Confidor) imidacloprid (100 mL) was applied to the soil of a rice plant and left to be absorbed. One strain (Nl9) was reared on rice plants that were treated with progressively higher concentrations (ranging from 10 to 180 mg L^{-1}) of imidacloprid over 13 generations. The other strain (Nl39) was reared directly onto rice plants treated with 200 mg L^{-1} of imidacloprid. A second culture of Nl9 and Nl39 was maintained on untreated rice plants.

3.2.4 Laboratory selection with ethiprole

Three strains (Nl33, Nl39 and Nl55) that demonstrated high levels of resistance to ethiprole were placed under further selection with ethiprole in the laboratory. These strains were reared on rice plants that were sprayed with progressively higher concentrations (ranging between 7.5 and 100 mg L^{-1}) of ethiprole (SC 200) over 5 generations. Populations were then reared onto a 100 mg L^{-1} rice plant every other generation. A second culture of Nl33, Nl39 and Nl55 was maintained on untreated rice plants.

3.3 Results and discussion

3.3.1 Imidacloprid resistance testing

Four of the strains had very high resistance (RR >160-fold) to imidacloprid (Table 3.1), whilst Nl55 displayed only moderate resistance. For all strains resistance was maintained in the absence of insecticide selection pressure for many generations suggesting that there was not a heavy fitness cost (under optimal laboratory conditions) involved in the resistance mechanism.

Table 3.1 Dose-response data for *N. lugens* laboratory susceptible and imidacloprid-resistant strains against imidacloprid topically applied to adults.

Compound	Strain	Generations without selection	LD_{50}-value [mg L^{-1}]	95% limits	Slope (± SD)	RR
Imidacloprid	Bayer-S	130	0.6	0.50-0.70	1.822 ± 0.158	1
	Nl9	12	97	3.40–434.00	0.762 ± 0.239	161.7
	Nl33	46	170.2	46.44-5095	0.655 ± 0.221	283.7
	Nl39	43	284.3	128.7-435.9	2.413 ± 0.698	473.8
	Nl40	42	119.9	nc	10.1 ± 20.7	199.8
	Nl55	32	14.49	4.55-30.81	0.653 ± 0.134	24.2

3.3.2 Ethiprole resistance testing

The earliest collected field strain (Nl9) showed high levels of resistance to ethiprole (Table 3.2), whilst the later collected strains demonstrated very high resistance (RR >300-fold). As seen with the imidacloprid bioassays, resistance to ethiprole in these populations can be maintained over many generations without further exposure to ethiprole. However, for Nl55 resistance reduced over time in the absence of further exposure to the insecticide. The second Nl55 bioassay (LC_{50} 2.74), was remarkably lower than the first bioassay (LC_{50} 112.7), with a four-generation gap between the two bioassays.

Table 3.2 Concentration-response data for *N. lugens* laboratory susceptible and fiprol-resistant strains against ethiprole applied as a leaf dip to adults.

Compound	Strain	Generations without selection	LC_{50}-value [mg L^{-1}]	95% limits	Slope (± SD)	RR
Ethiprole	Bayer-S	174	0.34	0.24-0.44	2.671 ± 0.432	1
	Nl9	2	25.56	5.23-62.57	1.125 ± 0.243	75.2
	Nl33	27	138.3	90.82-198.3	1.32 ± 0.148	406.8
	Nl39	22	337.6	140.9-1892	0.781 ± 0.149	993
	Nl40	25	312.4	nc	1.098 ± 0.541	918.8
	Nl55	18	112.7	54.03-281.8	0.693 ± 0.124	331.5
	Nl55	22	2.74	0.46-7.17	0.719 ± 0.141	8.1

3.3.3 Fipronil resistance testing

A second phenylpyrazole, fipronil, was tested against the *N. lugens* strains. Resistance to this alternative fiprol was present (Table 3.3), however, the level was markedly lower than seen for ethiprole, and this was common across all the strains. Nl33 and Nl39 (both Vietnam) displayed the moderate resistance (>30-fold), when compared to Bayer-S. Both strains had been previously exposed to fipronil spraying

in the field and it appears that the resistance has been maintained in the absence of insecticide pressure. Nl40 was remarkedly resistant to fipronil, when compared to the other field strains. It is not clear why this population was able to demonstrate high resistance in mortality bioassays, but was not possible to sustain a population in the laboratory when creating insecticide selected laboratory populations (described later, Section 3.3.5).

However, the value for Nl55 was considerably lower (RR 3-fold) than the other field strains. This mirrors the trend seen for Nl55 against ethiprole, since this population appears to have lost its resistance mechanism(s) to fipronil. Nl55 had also been exposed to fipronil spraying (two sprays) in the field, and so must have been able to survive field rates of fipronil at the time of collection. However, after 29 generations of non-selection in the laboratory, this resistance has broadly been lost. Again, Nl55 contrasts with Nl33 and Nl39 which have been able to maintain moderate levels of resistance to fipronil despite an even longer period of non-selection (> 44 generations). Unfortunately, no fipronil bioassays were conducted for Nl55 around the time of the 1st ethiprole bioassay (18 generations of non-selection), so we do not know if it had higher levels of resistance to fipronil then, before the sudden loss of ethiprole resistance.

Table 3.3 Concentration-response data for *N. lugens* laboratory susceptible and fiprol-resistant strains against fipronil applied as a leaf dip to adults.

Compound	Strain	Generations without selection	LC_{50}-value [mg L^{-1}]	95% limits	Slope (± SD)	RR
Fipronil	Bayer-S	174	1.16	0.70-1.66	10.858 ± 0.864	1
	Nl9	6	14.49	7.34-27.56	0.659 ± 0.078	12.5
	Nl33	46	37.13	1.06-137.3	1.259 ± 0.453	32.1
	Nl39	44	62.7	1.46-571	1.008 ± 0.412	54.1
	Nl40	51	295.1	176.4-3186	2.329 ± 0.974	254.4
	Nl55	29	3.46	0.77-8.21	0.966 ± 0.197	3

3.3.4 Laboratory selection with imidacloprid

To study the resistance mechanisms behind imidacloprid resistance, it was decided to put two of the field strains under further selection with imidacloprid. The strains, Nl9 and Nl39, were respectively selected with imidacloprid (by T.G.E. Davies) up to final concentrations of 180 and 200 mg L^{-1}. These selected populations (Nl9-imi and Nl39-imi) could then be contrasted with the unselected (Nl9 and Nl39) cultures of these strains.

3.3.5 Laboratory selection with ethiprole

Selections with ethiprole were also performed to increase resistance in the populations. This was attempted for four strains (Nl33, Nl39, Nl40 and Nl55), producing three populations of highly ethiprole resistant *N. lugens*. For the Nl40 population, despite showing high resistance in the bioassays, it was not possible to sustain a population treated with ethiprole. However, having three selected populations was deemed sufficient material for studying the mechanism(s) of ethiprole resistance.

3.3.6 Imidacloprid resistance of the laboratory insecticide selected strains

Selection of Nl9 and N39 with imidacloprid led to an increase in imidacloprid resistance in both populations (Table 3.4). The increase between Nl9 and Nl9-imi was modest (RR 162 versus 186), whereas for the Nl39-imi population the resistance to imidacloprid increased dramatically (LD_{50} >1000) compared with the unselected Nl39 populations (LC_{50} 284).

Interestingly the Nl33-eth population (LD_{50} 434) did see an increase in resistance to imidacloprid compared to the NL33 population (LD_{50} 170). However, this finding was not repeated for the Nl39-eth population, which is seen to be slightly less resistant than the Nl39 population (LD_{50} 284).

Despite the variation in response to insecticide selection across the populations there is a clear trend whereby all the populations kept under insecticide exposure throughout this PhD display very high resistance to imidacloprid when compared to the susceptible population.

Table 3.4 Dose-response data for *N. lugens* laboratory selected strains against imidacloprid topically applied to adults.

Compound	Strain	LD_{50}-value [mg L^{-1}]	95% limits	Slope (± SD)	RR
Imidacloprid	Nl9-imi	111.7	66.05-212	0.891 ± 0.11	186.2
	Nl39-imi	>1000	-	-	>1600
	Nl33-eth	433.8	101.3-14915	0.539 ± 0.161	723
	Nl39-eth	255.5	69.5-768	2.273 ± 0.854	425.8

3.3.7 Ethiprole resistance of the selected strains

Selection with ethiprole (Nl33-eth, Nl39-eth and Nl55-eth populations) led to a drastic increase in resistance to >14000-fold, compared to the susceptible Bayer-S strain (Table 3.5), in these populations. The resistance was so high that it was not possible to formulate LC_{50} values for these strains since there was negligible mortality, even at the highest dose (5000 mg L^{-1}) applied. For the Nl9-imi strain, that had not been selected with ethiprole, the resistance to ethiprole also markedly increased (LC_{50} 410) compared with Nl9 (LC_{50} 25.56). Such an increase in resistance to ethiprole, despite being selected with imidacloprid, could suggest there is cross resistance between the two compounds.

Table 3.5 Concentration-response data for *N. lugens* laboratory selected strains against ethiprole applied as a leaf dip to adults.

Compound	Strain	LC_{50}-value [mg L^{-1}]	95% limits	Slope (± SD)	RR
Ethiprole	Nl9-imi	410	273.3-1618	1.856 ± 0.580	>1200
	Nl39-imi	23.27	5.19-51.27	1.239 ± 0.303	68.4
	Nl33-eth	>5000	-	-	>14000
	Nl39-eth	>5000	-	-	>14000
	Nl55-eth	>5000	-	-	>14000

3.3.8 Fipronil resistance of the selected strains

The three ethiprole selected strains also displayed a large increase in resistance to fipronil, when contrasted with the unselected populations (Table 3.6), giving approximately 860-fold resistance when compared with the susceptible Bayer-S population. The Nl9-imi strain also saw a rise in fipronil resistance, compared with Nl9. However, the levels of resistance (56.4-fold) was far below those of the ethiprole selected strains. An interesting note from this is that selection with ethiprole clearly also causes increased resistance to fipronil. This led to the hypothesis that there

could be a resistance mechanism that could cause cross-resistance between these two compounds. Chapter V discusses these potential mechanisms involved in the resistance to fiproles in *N. lugens* and whether such a cross-resistance mechanism exists.

Table 3.6 Concentration-response data for *N. lugens* laboratory selected strains against fipronil applied as a leaf dip to adults.

Compound	Strain	LC_{50}-value [mg L^{-1}]	95% limits	Slope (± SD)	RR
Fipronil	Nl9-imi	65.340	26.41-157.3	1.016 ± 0.230	56.4
	Nl33-eth	>1000	-	-	>860
	Nl39-eth	>1000	-	-	>860
	Nl55-eth	>1000	-	-	>860

3.4 Conclusions

This chapter demonstrates the resistance of *N. lugens* to various insecticides that have been utilised for brown planthopper control. This builds on previous monitoring for field resistance that was conducted at Rothamsted Research (Gorman *et al.*, 2008). For all three compounds assessed (imidacloprid, ethiprole and fipronil) there was considerable resistance displayed in the field populations compared to the susceptible strain. This is clear from both the discriminating dose bioassays and the dose response bioassays. The high resistance to imidacloprid, ethiprole and fipronil demonstrated by the strains collected from 2010 onwards shows that these compounds are now mostly ineffective as control agents of *N. lugens* and should not be applied.

The most notable observation from the selection experiments described in this chapter was that selecting a population with ethiprole also increased resistance to fipronil. Given the structural similarity between these two phenylpyrazole

insecticides, it is not unsurprising that there appears to be cross-resistance between the two compounds. This has been seen previously when fipronil resistant *N. lugens* in China demonstrated high levels of cross-resistance to ethiprole (Zhao *et al.*, 2011). They found that selection with fipronil increased fipronil resistance (7.3-fold to 41.3-fold), but also caused an increase in ethiprole resistance (16.3-fold to 65.6-fold). A similar trend was found in the small brown planthopper, *Laodelphax striatellus*, with a laboratory-selected fipronil resistant strain showing moderate cross resistance to ethiprole (Wei *et al.*, 2016). This finding is of field relevance because it suggests that fipronil would not be a viable replacement for ethiprole (and vice versa) in regions where ethiprole (or fipronil) was no longer able to demonstrate control of *N. lugens* within economic thresholds.

The finding that most populations, except for NI55, maintain high resistance to the test compounds, in the absence of further exposure to insecticide is a key finding. There is a widely held assumption that resistance to xenobiotics will carry a fitness cost in the absence of xenobiotics (ffrench-Constant and Bass, 2017). For *N. lugens* there was a link between imidacloprid resistance and fitness. A highly imidacloprid resistant strain had clear reproductive disadvantages when compared to a susceptible strain, with the authors concluding that absence of imidacloprid would quickly cause the population to regain imidacloprid sensitivity (Liu and Han, 2006). Reasons for supposed fitness cost would be that producing high levels of detoxification enzymes would have a significant energy cost (Kliot and Ghanim, 2012; ffrench-Constant and Bass, 2017). Target-site mutations (*kdr* and *Rdl*) have been linked with fitness cost in *Anopheles gambiae* (Platt *et al.*, 2015), but it has also been suggested that A301S has no significant fitness cost due to its ability to persist in

absence of xenobiotic selection (Thompson, Steichen and ffrench-Constant, 1993; Aronstein, Ode and ffrench-Constant, 1995; Holbrook *et al.*, 2003; Bass *et al.*, 2004). The clear majority of research conducted on fitness costs is performed in a laboratory, rather than field, setting so is difficult to predict the impact of insecticide resistance related fitness costs on field populations of pests. The fact that three of the strains tested here (Nl33, Nl39 and Nl40) showed an ability to maintain resistance in absence of insecticide exposure in the laboratory, does not mean the same finding would occur in the field. However, if the costs associated with the insecticide resistance seen here are minor then this has important implications for insecticide resistance management strategies. Since resistance has already evolved to these compounds, one of the aims of any IRM programme would be to attempt to regain lost susceptibility of *N. lugens* to these insecticides. However, if it is possible to maintain resistance to insecticides in the field (in the absence of exposure to these insecticides) then it may prove unviable in the long term to try to reintroduce these compounds for control.

Understanding the mechanisms that lie behind insecticide resistance is a core concept behind resistance management strategies urgently needed to control brown plant hopper. This allows for diagnostics to be developed to assess how resistant a field population of *N. lugens* might be; crucial information that can lead to a more sustainable programme of insecticide application. This is needed, due to the simple fact that it is inefficient to apply insecticides that will not be able to provide control, and only exacerbates the problem of insecticide resistance.

Therefore, insecticide resistance management strategies for *N. lugens* are a mix of sustainable pesticide use, biological control and resistant rice varieties. It has been argued that pesticide use should not be a core component of IPM strategies for rice, but instead allow natural enemies to confer biological control (Matteson, 2000). Since there is now widespread resistance to insecticides there is interest in other means of containing *N. lugens* outbreaks, specifically parasitoids and predators (Gurr *et al.*, 2011). Unfortunately the extensive use of pesticides has had a large detrimental effect on natural enemy populations (Matteson, 2000; Bottrell and Schoenly, 2012). However it has been shown by IRRI that effective landscape management and reduced pesticide use can cause control of insect pests of rice by allowing natural enemies to thrive (Settele, Biesmeijer and Bommarco, 2008; Gurr *et al.*, 2011). However, implementing such strategies across such a large geographical area are complex and so judicious use of pesticide is still recommended by IRAC for rice hopper control, as well as the biological control discussed here.

The potential mechanisms that could underlie the resistance discussed in this chapter, will be discussed in chapters IV-VI.

Chapter IV Generation of a new *de novo* transcriptome for analysis of insecticide resistance in *N. lugens*

4.1 Introduction

The advent of next generation sequencing has drastically transformed the field of molecular biology (Behjati and Tarpey, 2013) and revolutionised the methods of studying insecticide resistance. The ability to sequence an insect's entire mRNA, and therefore all its transcripts, at the point of collection, allows for the generation of a transcriptome. This represents a valuable resource for researchers working on the topic of insecticide resistance and can be mined for candidate genes or genetic variation potentially associated with resistance.

RNA-seq (RNA sequencing) is the most widely used method to sample gene transcripts, replacing previous approaches such as hybridisation-based microarrays (Kukurba and Montgomery, 2015). The advantages of RNA-seq over previous technologies are numerous: no prior knowledge of gene/ transcript sequences are required, increased detail of gene expression, analysis of alternate splicing and no issues with cross-hybridisation artefacts (Kukurba and Montgomery, 2015). The ever-increasing affordability of RNA-seq, as sequencing technology has improved, has allowed it to become commonplace in the study of insecticide resistance.

At the start of this PhD in 2013, there were already some genetic resources available for *N. lugens*. Although, there was no draft or reference genome for *N. lugens* available at this point the transcriptomic resources that were available covered a broad range of life stages and tissues of *N. lugens*. The first *N. lugens*

genomic resource contained 37,000 ESTs (Noda *et al.*, 2008) and this was later incorporated into a transcriptome pyrosequencing assembly of 8,911 contigs and 10,620 singletons (Bass, Hebsgaard and Hughes, 2012). A further three unique transcriptomes had been published, generated from different tissues: midgut (Peng *et al.*, 2011), intestine (Bao *et al.*, 2012) and salivary glands (Ji *et al.*, 2013). December 2013 saw the publishing of another *N. lugens* transcriptome of whole insects at different life stages (Wan *et al.*, 2014). In 2015, a genomic and transcriptomic study was published analysing the P450 gene family in *N. lugens* (Lao *et al.*, 2015). This involved the application of sub-lethal doses of imidacloprid, triazophos and deltamethrin respectively before performing RNA-seq. This found that there was significant overexpression (>3-fold) for *CYP6CS1v2* and *CYP4CE1v2*, whislt *CYP4DE1, CYP417A1v2* and *CYP439A1* were >2-fold expressed compared to the control.The end of 2014 saw the publication of the first *N. lugens* genome (Xue *et al.*, 2014). This produced an assembled genome size of 1.14 Gb, with 27,571 protein coding genes of which 10,245 had gene ontology (GO) terms. This assembled genome had a contig N50 of 24.2 and a scaffold N50 of 356.6.

This chapter highlights the creation of a *de novo* transcriptome which was used to analyse differential gene expression between insecticide susceptible and resistant strains. This transcriptome also allowed the manual curation of several insecticide target sites which could then be characterised in more detail, discussed in chapters five and six.

4.2 Material and methods

4.2.1 Gene assembly from bioinformatics resources

Galaxy and Geneious were used to assemble partial genes from single read archives (SRA). The bioinformatics department at Rothamsted Research kindly downloaded the required SRA datasets and normalised them, before uploading them to Decypher. This is a dedicated server for DNA/protein similarity searches and was built and supported by Timelogic (Carlsbad, CA, USA). Conserved AA sequences could then be blasted against these SRA datasets, using tera-blastx (AA versus translated nucleic) to return matching hits. These hits were then downloaded into Geneious and *de-novo* assembly performed to return consensus sequences. These sequences were then set as references and mapped against the NL SRA database to extend the sequences. *De novo* assembly of the reads returned from this extended mapping was performed and the resulting consensus sequence was analysed for potential ORFs which could be mapped to a reference sequence.

4.2.2 Illumina sequencing

Total RNA was extracted from pooled homogenates of six insects from Bayer-S, Nl33, Nl55, Nl33-eth and Nl55-eth (4 biological replicates per strain) using the Bioline Isolate RNA mini kit (Chapter II, Section 2.8). Total RNA was used as a template for the generation of barcoded libraries (TrueSeq RNA library preparation, Illumina). These libraries were sequenced by The Genome Analysis Centre (TGAC, Norwich, UK) with replicates multiplexed for sequencing on an Illumina HiSeq 2500 flowcell (100 bp paired reads) to generate at least 15 million reads per biological replicate. FastQC (version 0.11.2) was used to check the quality of the raw reads obtained, and reads

were trimmed of low quality sequence and adapter sequence using Trimmomatic (Bolger, Lohse and Usadel, 2014).

4.2.3 Trinity *de-novo* assembly

Trinity is a method designed for assembling RNA-seq data into a transcriptome without the need for a reference genome (Grabherr *et al.*, 2011). Trinity is a combination of three independent software modules: Inchworm, Chrysalis and Butterfly. Inchworm is the first step to be applied to the RNA-seq raw reads and it assembles transcripts using a *k*-mer based approach. This entails establishing each read as a set of overlapping *k*-mers (default *k* = 25), and removing *k*-mers that likely contain sequencing errors. A seed *k*-mer (the most abundant *k*-mer) is set as the basis for constructing transcripts. This is done by a greedy expansion based on frequencies of overlapping *k*-mers. This continues until no *k*-mers are left and full length transcripts for dominant isoforms are reported. This data is then used by Chrysalis to create de Bruijn graphs from clusters of the minimally overlapping Inchworm contigs. Finally, Butterfly is run to reconstruct full length transcripts from the de Bruijn graphs generated by Chrysalis. This is broken down into two phases, with the first phase being graph simplification by trimming the edges in the de Bruijn graph. The second phase is defined as plausible path scoring. This is where the paths (possible transcripts) are analysed to see which are plausible based on the reads that assign to them. This then produces a list of linear transcripts, with information on spliced isoforms provided. This program was run on a linux server at Rothamsted Research by sending command line prompts using UNIX commands. The Trinity parameters were paired ends mode and the CPUs assigned were 2 for Inchworm and 32 for Butterfly.

4.2.4 Annotation of transcriptome

The Galaxy server was used to run a tera-blastx search against the non-redundant protein database (NCBI 06/01/15). The parameters of this search were an e-value of 1 x 10^{-3} and a minimum score of 50. This was performed for the combined transcriptome assembly (assembled from all five populations sequenced). This analysis produced an XML file that was imported into Blast2GO (Conesa *et al.*, 2005) to process the blast results from Galaxy. Contigs of interest identified from this were then imported into Geneious for further analysis.

4.2.5 Calculating differentially expressed genes between *N. lugens* populations

The de novo assembled transcriptome was used as a reference to map raw reads and quantify levels of gene expression of assembled transcripts. RNA-seq by Expectation Maximization (RSEM) was used for this, since it has the major benefit of not needing a reference genome, and can decipher transcript abundancies solely from RNA-seq data (Li and Dewey, 2011). RSEM utilises Bowtie, an ultrafast alignment program (Langmead *et al.*, 2009), to align the reads to the transcripts and present an estimate of each transcript's expression level. This data was then processed using two individual packages designed to identify transcripts that have been differentially expressed: EdgeR (FDR 0.05, fold change <0.5 and >2)(Robinson, McCarthy and Smyth, 2009) and DESeq2 (Love, Huber and Anders, 2014). Both software programmes work on a negative binomial model for analysing expression. EdgeR is similar to a previous procedure, Limma (Smyth, 2004), designed for differential expression analysis. EdgeR uses the table of counts produced from RSEM, and an over dispersed Poisson model, and can separate biological and technical variation. Gene-wise dispersions are calculated by conditional maximum likelihood,

with an empirical Bayes procedure used to create a consensus value. The differential expression value given for each gene is derived from an exact test (analogous to Fisher's exact test) that has been modified for over dispersed data (Robinson, McCarthy and Smyth, 2009). DESeq2 differs from EdgeR in its method of estimating the gene dispersal. DESeq2's methodology is advantageous for experiments with small sample sizes. This is because smaller sample sizes can cause high levels of variation in dispersal estimates per gene. DESeq2 solves this problem by sharing information across genes, with the underlying assumption that genes with similar expression strength will have similar dispersion (Love, Huber and Anders, 2014). This then gives a more accurate gauge of fold change for genes.

4.2.6 GO enrichment analysis

To attempt to define the biological properties (cellular component, biological process and molecular function) of the differentially expressed transcripts, GO-term enrichment was performed. The enrichment analysis of GO-terms (for the differentially expressed transcripts), was conducted using Fisher's exact test within Blast2GO software with a False Discovery Rate of <0.05. The GO-terms of the differentially expressed transcripts were compared to the GO-terms of the reference transcriptome (assembly 1). This was performed separately for the six sets of differentially expressed transcripts derived from the EdgeR and DESeq2 analysis.

4.2.7 Database submission

Sequence data used in this study have been deposited at the National Center for Biotechnology Information as follows:

BioProject (accession no PRJNA331084)

BioSample (accession numbers SAMN05437238, SAMN05437239, SAMN05437240, SAMN05437241, SAMN05437242)

Run (accession no SRP079631)

4.3 Results and Discussion

4.3.1 Sequencing and *de novo* transcriptome assembly

Total RNA from five populations of *N. lugens* (four biological replicates per population) were sequenced using paired-end Illumina. This produced a total of approximately 964 million 100 bp long reads, which were then used to create a reference transcriptome (Table 4.1, assembly 1). FastQC was performed prior to assembly to assess base quality and all samples scored greater than 30. A non-normalised assembly was run using Trinity, which produced 233,800 contigs of an average length of 833 bp, of which 169,709 were denoted as Trinity 'genes'. This is clearly far higher than the number of genes predicted from the genome assembly (27,571 protein coding genes). The availability of the *N. lugens* genome had led to hopes that genome guided trinity transcriptome assembly could be performed. This should have significantly improved identification of full length transcripts from the RNA-seq data, which remains complex (Boley *et al.*, 2014). However, multiple attempts to perform this pipeline were unsuccessful. It appeared that the GFF file available was unusable for our purposes, and attempts to create our own one was thwarted by a lack of local computing power. Although six assemblies were created from the RNA-seq data, full statistics given in Table 4.1, the assembly that was predominantly used was assembly 1. This was because it was assembled using all the

raw reads, therefore providing the most complete reference transcriptome to use for differential gene expression analyses.

Table 4.1 A comparison of transcriptome assemblies.

Assembly ID	1	2	3	4	5	6
Population(s)	Combined	Bayer-S	Nl33	Nl55	Nl33-eth	Nl55-eth
Raw reads	963,705,042	198,351,440	179,331,150	179,451,430	217,279,840	189,291,182
Mean Q30 to base	101	101	101	101	101	101
Contigs	233,800	121,710	133,027	127,221	138,082	128,325
Trinity 'genes'	169,709	93,308	100,757	95,863	104,301	97,158
GC content	39.7	39.6	39.7	39.8	39.4	39.2
Min contig length	201	201	201	201	201	201
Max contig length	26,366	26,929	25,646	26,089	26,948	26,953
Mean contig length	833	901	906	900	915	879
Median contig length	393	435	439	449	440	434
N50	1,645	1,728	1,730	1,683	1,765	1,640

4.3.2 Annotation of the reference transcriptome

A blast analysis of the *N. lugens* sequences produced by the Trinity assembly revealed that 21% (49118) of the contigs had at least one hit against the NCBI nr protein database. These contigs had an average read length of 2063 bp. The remaining 79% of contigs (average read length 506 bp) that could not be assigned a BLAST hit, highlight the limitations of a BLAST tool for analysing transcriptomes. Karatolos et al discuss these limitations (Karatolos *et al.*, 2011). It is likely that ongoing sequencing projects on related insect species will allow improved future annotation of these genes.

The number of contigs in the assembled transcriptome with assigned BLAST hits is considerably higher than the number of genes predicted by the genome annotation, this is most likely a result of multiple contigs being generated for many genes depending on alternate splicing/allelic variation. Another reason for the high number of contigs would be the low quality of the assembly, with a large number of small contigs being assembled that are unlikely to have any biological significance.

4.3.3 GOIs for metabolic resistance

The primary reason for conducting this RNA-seq experiment was to study genes encoding potential insecticide detoxification enzymes. The gene families of key interest were the P450s, CCEs and GSTs and these were manually curated from the transcriptome. Within the Trinity transcriptome there were 220 contigs that returned BLAST hits to P450s, 49 to CCEs and a further 31 to GSTs. The *N. lugens* genome study published in 2014 provided an extensive comparison of these detoxification families between *N. lugens* and various other insect genomes (Xue *et al.*, 2014). They highlight

that there are 67 genes encoding P450s in *N. lugens* and only 11 encoding GSTs, which is significantly fewer than that seen for most other insect species studied. Of those 67 P450 found in the genome, the trinity assembly returned full-length sequence for 48 of these and partial for a further six (Table 4.2) However, when performing a *de novo* assembly of the 67 P450s from the genome, the result is 55 unique sequences, meaning the trinity assembly has very good coverage of the P450 repertoire. The GSTs coverage of the trinity assembly assembles 7/11 sequences from the genome. As for the CCEs, the nine available sequences in GenBank all *de novo* assemble into one contig. However, none of the 49 transcripts assigned with CCEs BLAST hit align to this sequence.

A further study published in 2015 gave an overview of the cytochrome P450 monooxygenase repertoire in *N. lugens* (Lao *et al.*, 2015) and also suggested reasons for the paucity of *N. lugens* P450 genes. They argue that since *N. lugens* is a monophagous (rice phloem sap) pest, it does not encounter as many plant secondary metabolites as more polyphagous insect species and therefore does not require such a large pool of detoxification genes. As mentioned in section 4.3.3 the high number of contigs matching P450s is probably due to allelic variation, multiple isoforms of many genes and poor quality assembly.

Table 4.2 A summary of the detoxification genes (P450s, GSTs and CCEs) found in the Trinity assembly compared to the *N. lugens* genome.

		P450s	GSTs	CCEs
	Genome	67	11	9
Trinity assembly	Full sequence	48	6	0
	Partial sequence	3	1	1

4.3.4 Screening for differentially expressed transcripts

Extensive gene expression analysis was conducted to identify candidate genes that could potentially be involved in metabolic resistance to xenobiotics. The assembled transcriptome was used as a reference for the Illumina raw reads to be mapped against using bowtie. This provided count matrices of abundance for the transcripts for all 20 biological replicates. Two programmes were used for estimating differential expression, EdgeR and DESeq2. A false discovery rate of < 0.05 for EdgeR and a padj of < 0.05 for DESeq2 was used. The transcripts from both analyses were merged to show which transcripts were DE in both tests (Table 4.3), which revealed that a huge number of transcripts were differentially expressed between *N. lugens* populations. Between Bayer-S and the two ethiprole selected populations, Nl33-eth and Nl55-eth, there were 11455/6806 (Fig. 4.1) transcripts showing > 2-fold expression respectively. Even between a selected and unselected population (Nl33 and Nl33-eth) there were a high number (2634, FC > 2) of DE transcripts (Fig. 4.2). The only exception to such large numbers of DE transcripts was in the comparison between Nl55 and Nl55-eth that saw a moderate level of 161 (FC > 2) DE transcripts (Fig. 4.2). For a summary of the top 20 expressed transcripts in each comparison see the appendix (Tables A9-14).

Table 4.3 A summary of transcripts differentially expressed in both EdgeR and DESeq2.

	FDR 0.05		FDR 0.01	
	FC <0.5 & >2	FC > 2	FC <0.5 & >2	FC > 2
Bayer-S vs Nl33	12448	7111	8041	4696
Bayer-S vs Nl33-eth	18169	11455	11790	7806
Nl33 vs Nl33-eth	3852	2634	1992	1437
Bayer-S vs Nl55	10898	6014	6691	3839
Bayer-S vs Nl55-eth	11513	6806	7412	4601
Nl55 vs Nl55-eth	227	161	112	39
In all*	703	640	417	394

*Excluding NI55 vs NI55-eth

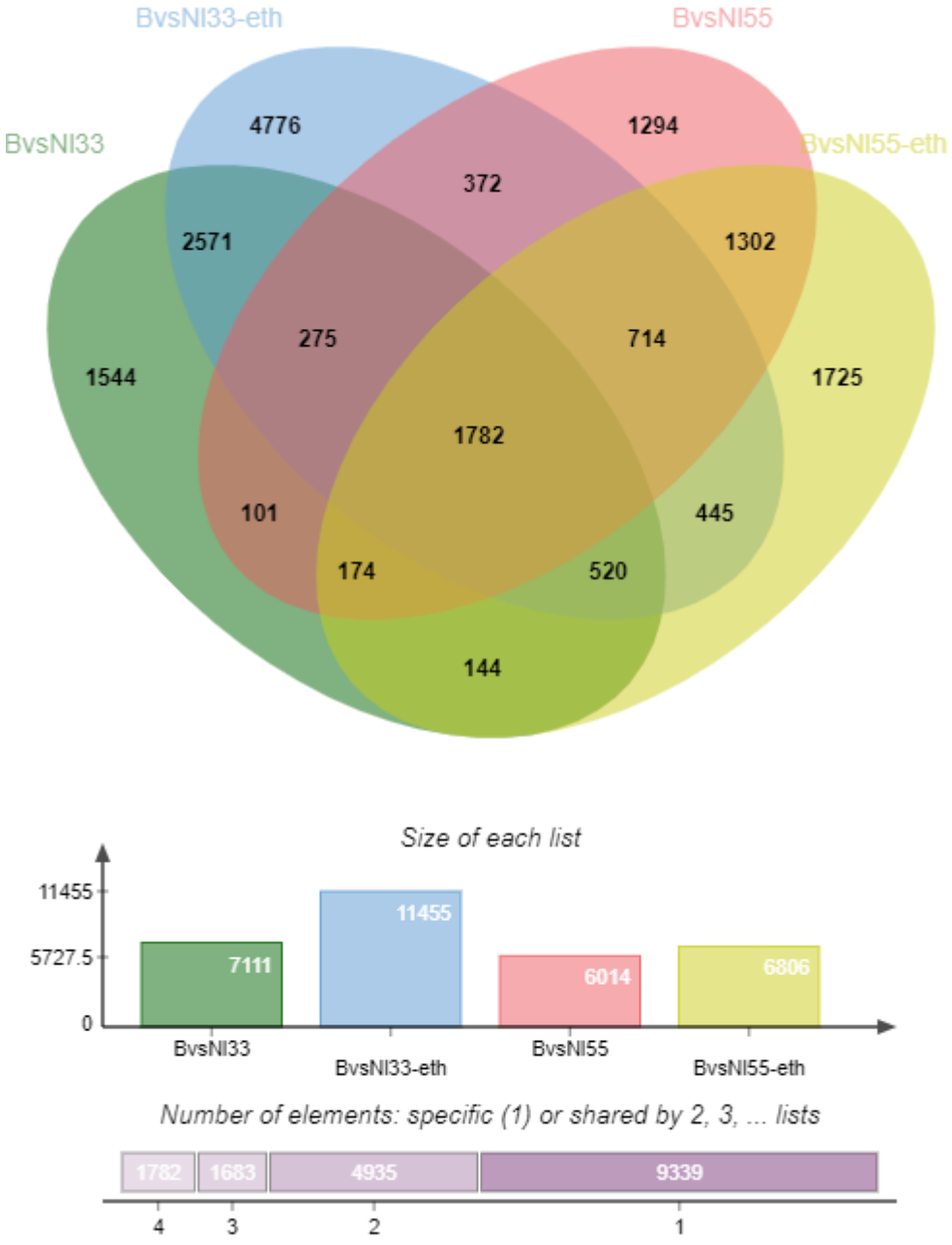


Fig. 4.1 Top: Venn diagram showing differentially expressed transcripts in multiple RNA-Seq comparisons of susceptible vs resistant *N. lugens* (B=Bayer-S). Bottom: the number of differentially expressed transcripts (FC > 2) in each pairwise comparison.

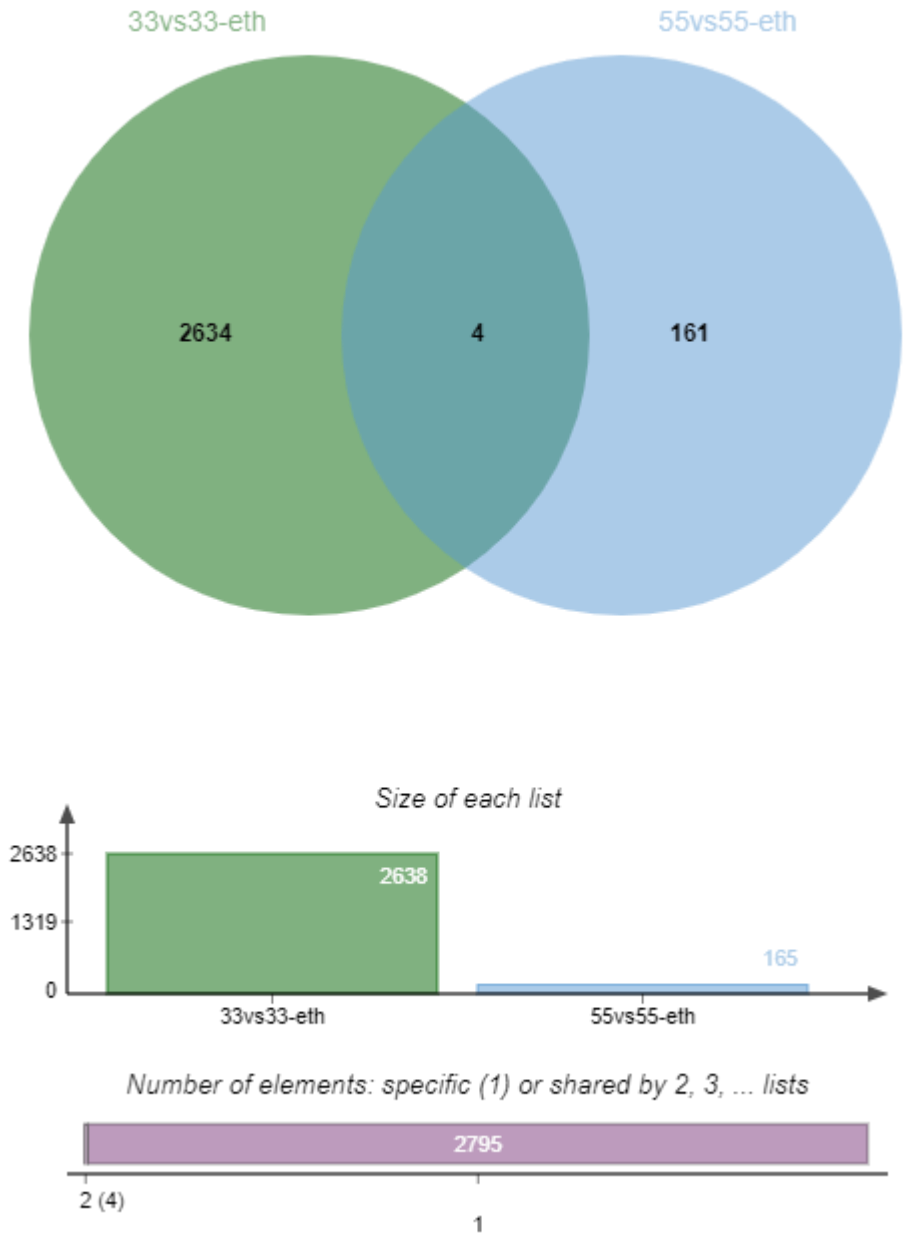


Fig. 4.2 Top: Venn diagram showing differentially expressed transcripts (FC > 2) in RNA-Seq between unselected and selected populations of N. lugens. Bottom: differentially expressed transcripts in each comparison.

Such levels of DE transcripts were far above the levels we were expecting to see when this RNA-Seq experiment was designed. There are a few possible reasons for these high gene counts. Firstly, there may be significant genetic variation between

strains due to length of time since field collection and location of origin. The susceptible reference strain (Bayer-S) is from Japan, NL55 is from India and NL33 is from Vietnam. Furthermore, Bayer-S has been laboratory reared for an extended period (> 15 years). During this time is it plausible that a significant number of the genes that are still essential for *N. lugens* in the field, are no longer being expressed in the laboratory populations. Secondly, as previously discussed, there are a huge number of transcripts in this transcriptome compared to predicted gene number. It is possible that allelic variants that are categorised as different isoforms could be skewing the number of transcripts that are genuinely differentially expressed.

Gene enrichment analysis was performed but ultimately proved unsuccessful at revealing which GO terms were enriched in the differentially expressed transcript sets against the reference transcriptome dataset. At an FDR of < 0.05 there were no statistically significant enriched GO terms returned for Bayer-S vs Nl33-eth, Nl33 vs Nl33-eth and Nl55 vs Nl55-eth. For the remaining three experimental comparisons (Bayer-S vs Nl33, Bayer-S vs Nl55 and Bayer-S vs Nl55-eth) there were some GO terms enriched. However, these were all for viral processes (data not shown) and were seen as having limited relevance to our study of insecticide resistance.

Despite the large number of transcripts being shown as DE, it was possible to narrow down candidate genes by looking for transcripts that were DE in all experimental conditions in both EdgeR and DESeq2 (Table 4.3). Nl55 vs Nl55-eth was excluded from this analysis due to the very low number of DE genes in this comparison. If Nl55 vs Nl55-eth was left in this analysis then there were only three

DE transcripts, none of which were strong candidates for a role in resistance. This analysis produced 703 transcripts, of which 257 were assigned a BLAST hit.

Of the 257 differentially expressed transcripts, three were identified as putative candidate genes in xenobiotic resistance. However, I included a further five candidate genes (all carboxylesterases) since they were all overexpressed in EdgeR analysis, and overexpressed in most experimental conditions in the DESeq2 analysis. We decided it was necessary to analyse these as well, as a previous study had implicated esterases in ethiprole resistance in *N. lugens* (Punyawattoe *et al.*, 2013). This narrowing of candidate genes assumes that the same resistance mechanism(s) caused resistance in all the resistant strains. Table 4.4 gives an overview of the candidate detoxification genes identified from the combination of EdgeR and DESeq2 analysis. The fold change values displayed are for those from the EdgeR analysis. All eight transcripts are significantly overexpressed compared to the Bayer-S strain, and show low levels of overexpression when comparing Nl33 to Nl33-eth. The eight transcripts are formed of five carboxylesterases, two P450s and one multidrug resistance-associated protein (ABC transporter). Of this one gene was already known to play a role in insecticide resistance in *N. lugens*, c103228_g2_i1, encoding the P450 enzyme, CYP6ER1. This is an enzyme that had previously been linked to imidacloprid resistance in *N. lugens* by overexpression of the gene (Bass *et al.*, 2011). Chapter VI focuses in detail on the analysis of this gene, however it is included here as it represents a useful positive control for our pipeline/analysis.

Table 4.4 Genes of interest (GOIs) after differential expression analysis. FC = Fold change in expression.

Transcript	Seq. description	FC using EdgeR				
		Bayer-S vs Nl33	BayS vs Nl33-eth	Nl33 vs Nl33-eth	BayS vs Nl55	BayS vs Nl55-eth
c91313_g1_i1	Multidrug resistance-associated protein	193.2	808.4	3.6	401.0	494.6
c91960_g1_i2	Carboxylesterase-6	195.7	1884.5	3.4	443.2	976.6
c98716_g1_i1	Carboxylesterase-6	1159.8	11676.1	3.5	2462.4	4258.6
c101335_g1_i1	Carboxylesterase, partial	263.2	4365.1	5.3	805.7	2299.7
c103228_g2_i1	Cytochrome P450 *CYP6ER1*	70.2	49.4	2.9	24.3	68.7
c103516_g1_i4	Carboxylesterase, partial	2119.5	12197.2	18.8	2098.2	6733.6
c103516_g1_i6	Carboxylesterase, partial	185.6	1949.4	3.5	415.4	782.2
c103740_g1_i2	Cytochrome, partial	2000.0	9217.3	4.3	4669.9	5334.9

These candidate genes were then further analysed by qRT-PCR (primers, Appendix A, Table A2), to correlate with the EdgeR/DESeq2 analysis. Bayer-S was used as the reference strain for the qRT-PCR relative gene expression analysis. Again, all the candidate genes were highly overexpressed when compared with the reference (Table 4.5). Transcripts c101335_g1_i1 (partial CCE) and c103228_g2_i1 (*CYP6ER1*) are not included in this analysis. This is because there was no expression of c101335_g1_i1 in Bayer-S so it was not possible to generate a relative gene expression value for the other strains. For c103228_g2_i1, extensive qRT-PCR studies are described in chapter VI, and as it was a known candidate gene it did not need further validation here.

Although the qRT-PCR studies on the candidate genes appeared to correlate with the DE analysis conducted on the RNA-Seq data, a few problems arose. Firstly, the 95% confidence limits were very large in most of the populations tested. This was due to a large amount of variation in gene expression level between biological replicates of the populations. If overexpression of a gene was genuinely involved in insecticide resistance, then the expectation would be that the variation in expression would reduce after insecticide selection, manifested by reduced 95% confidence limits (Garrood *et al.*, 2016). Secondly the levels of expression in the reference strain

(Bayer-S) were very low, and it is possible that there is no or only very limited expression in Bayer-S and this skews the analysis, resulting in huge overexpression values.

Therefore, it was decided to go back to the original FPKM (Fragments Per Kilobase Million) values generated from the bowtie mapping of raw reads to the transcriptome. The average FPKM values (average of four biological replicates) are displayed for the five populations sequenced for the eight candidate genes (Table 4.6). This showed why such huge overexpression values were being generated in the EdgeR/DESeq2/qRT-PCR by revealing negligible FPKM values for seven out of eight of the candidate genes in Bayer-S, which then caused the misleading overexpression values for the resistant populations. Of the eight candidate genes, only one appeared to be a genuine candidate after the FPKM analysis, and this was c103228_g2_i1 (*CYP6ER1*), which had already been previously identified as a candidate gene in imidacloprid resistance (see above). However, the FPKM values of this gene are a useful benchmark for comparing the other candidate genes too. Since the FPKM values of c103228_g2_i1 here demonstrate the levels of expression that would be expected in a gene causing insecticide resistance, it was used to compare to the other candidate genes. None of the other candidate genes' FPKM values even came close to c103228_g2_i1 values. The highest value seen is 61.18 for c103516_g1_i6 in the

Nl33-eth population, but this is still significantly lower than the value for c103228_g2_i1 in Nl33-eth (522).

So, it was decided that none of the candidate genes, except c103228_g2_i1, were strong candidates for an involvement in insecticide resistance via gene overexpression.

Table 4.5 Fold change in expression of GOIs in multiple populations of *N. lugens* when compared with the susceptible reference strain as determined by quantitative real time PCR.

		Bayer-S		Nl33		Nl55		Nl33-eth		Nl55-eth	
Transcript	Seq. description	2^-ΔΔCt	95% conf. limits	2^-ΔΔCt	95% conf. limits	2^-ΔΔCt	95% conf. limits	2^-ΔΔCt	95% conf. limits	2^-ΔΔCt	95% conf. limits
c91313_g1_i1	Multidrug resistance-associated protein	1.1	0.43	39.3	10.9	89.4	109.8	171.3	81.1	95.4	73.9
c91960_g1_i2	Carboxylesterase-6	1.2	0.9	93.9	75.1	564.3	508.1	1870	1026	911	748
c98716_g1_i1	Carboxylesterase-6	1.1	0.5	19	18.4	148.7	138.3	657	377	225.7	195.6
c103516_g1_i4	Carboxylesterase, partial	1.1	0.6	27.1	22.5	250	230	920	540	477	429
c103516_g1_i6	Carboxylesterase, partial	1.1	0.6	1565	1369	388222	741005	42200	28634	12323	10732
c103740_g1_i2	Cytochrome, partial	1.3	1	153.2	66	342	321	701	479	545	472

Table 4.6 Average FPKM values per *N. lugens* population for the GOIs from the RNAseq experiment.

		FPKM				
Transcript	Seq. description	Bayer-S	Nl33	Nl55	Nl33-eth	Nl55-eth
c91313_g1_i1	Multidrug resistance-associated protein	0.03	6.50	12.81	26.10	15.86
c91960_g1_i2	Carboxylesterase-6	0.02	4.69	9.74	43.65	22.02
c98716_g1_i1	Carboxylesterase-6	0.00	3.51	7.56	33.10	12.96
c101335_g1_i1	Carboxylesterase, partial	0.00	2.41	7.29	37.02	20.77
c103228_g2_i1	Cytochrome P450 CYP6ER1	7.10	192.20	519.22	522.66	362.54
c103516_g1_i4	Carboxylesterase, partial	0.00	8.50	8.51	45.65	27.09
c103516_g1_i6	Carboxylesterase, partial	0.02	6.07	12.53	61.18	23.92
c103740_g1_i2	Cytochrome, partial	0.00	4.10	10.06	18.01	11.19

4.3.5 Assembly of insecticide target sites from available transcriptomes

4.3.5.1 Glutamate-gated chloride channel assembly

As detailed in the introduction, several transcriptomes were available to mine for potential genes involved in detoxification of xenobiotics when this PhD was started. However, the quality of the assembled transcriptomes was too low to find full length genes of interest. This was demonstrated by analysis of the transcriptome, specifically looking for genes involved in detoxification (Bao *et al.*, 2012). There were only three unigenes covering the GABA-gated chloride channel and one unigene for the glutamate-gated chloride channel (151 bp).

Therefore, an alternative approach was taken, going back to the publicly available SRA raw data that the transcriptomes were annotated from. The bioinformatics department at Rothamsted created a normalised database of all the available SRA files for *N. lugens*, which contained 31,743,591 single reads. This allowed highly conserved AA sequences to be aligned using tera-blastx against this archive to return nucleotide sequences that could be assembled into longer reads.

The first gene this was performed for was the glutamate-gated chloride channel. The glutamate-gated chloride channel of several other species of insect (*Apis mellifera*, *Laodelphax striatellus*, *Nasonia vitripennis*, and *Plutella xylostella*) were used to BLAST the SRA database. This returned 964 reads, which after assembly and further mapping yielded a gene of 1791 bp with a putative CDS of 452 AAs. Validation of this gene was carried out by designing primers from this sequence (Appendix A, Table A3) and sequencing the channel in the four ethiprole resistant

strains and the susceptible strain. Fig. 4.3 shows the CDS and transmembrane regions.

Fig. 4.3 Amino acid alignment of *N. lugens* glutamate-gated chloride channel. Also shown are the glutamate-gated chloride channel sequences of *A. mellifera* (accession NM_001077809.1), *L. striatellus* (JQ413991.1), *N. vitripennis* (FJ851099.1) and *P. xylostella* (JX014231.1). The signal peptide and four transmembrane regions are highlighted.

4.3.5.2 GABA-gated chloride channel assembly

The *N. lugens Rdl* gene (encodes the GABA-gated chloride channel) was already available from GenBank (accession no KC841916). However, this was used to blast against the SRA database to search for any underlying variation in the gene which could be potentially be involved in fiprole resistance.

This revealed an isoform (Nl RDL 9B) which is shown in Fig. 4.4, aligned with the publicly available sequence. The novel isoform was submitted to GenBank

(accession no KX592155). Although the nucleotide sequence differed considerably between the two variants after the third transmembrane region, there is only a single amino acid change in the final transmembrane region. One isoform expresses a methionine and the other a valine. A more thorough analysis of this gene and the consequences for fiprole insecticides efficacy is discussed in chapter V.

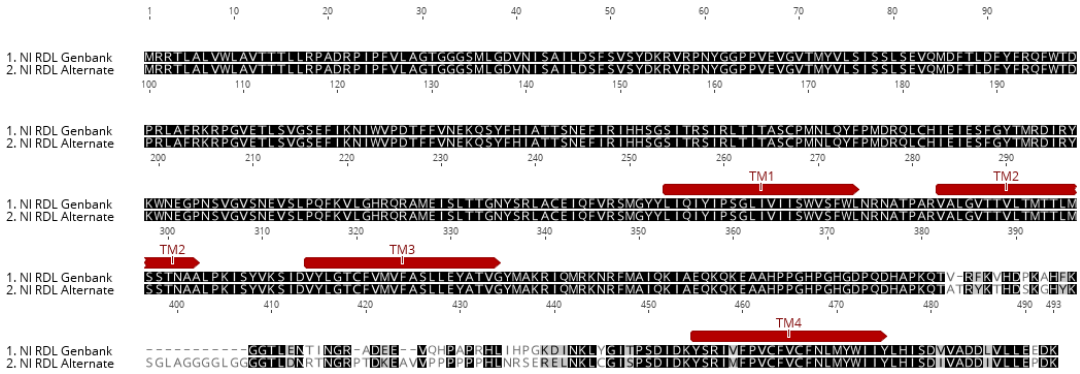


Fig. 4.4 Amino acid alignment of *N. lugens* RDL subunit sequences with the top sequence displaying exon 9A and the bottom sequence exon 9B. The four transmembrane regions are highlighted.

4.3.5.3 Voltage-gated sodium channel assembly

The procedure for obtaining the full-length sequence of this gene was the same as for assembling the GluCl gene. VGSC's from four species (*Musca domestica, P. xylostella, N. vitripennis* and *Tribolium castaneum*) were aligned using tera-blastx against the SRA database. This returned 1384 reads, which after assembly and mapping gave almost the entire VGSC sequence (with a small gap). Primers were designed (Appendix A, Table A3) to this retrieved sequence and sequencing VGSC using cDNA isolated from Bayer-S allowed this gap to be bridged and the full VGSC assembled (Appendix A, Table A3).

The resistance mechanism that was thought most likely to be responsible for pyrethroid resistance was knockdown resistance (*kdr)*, a commonly seen target site

mutation (Williamson *et al.*, 1993, 1996). The sequencing of the VGSC searched for all the mutations that have been linked with causing pyrethroid resistance in various pest species (Rinkevich, Du and Dong, 2013). Somewhat surprisingly there was not a single mutation that has been linked with pyrethroid resistance seen in any of the deltamethrin resistant strains (bioassay data not shown) that were sequenced. Furthermore, since pyrethroids have now been banned from use against *N. lugens*, there is little field relevance to continued work on *N. lugens* pyrethroid resistance.

4.3.6 Transcripts encoding insecticide targets

The second goal of the RNA-seq study was to identify transcripts encoding insecticide target sites, that could then be screened for potential resistance causing mutations. Given this PhD focuses on the neonicotinoid and phenylpyrazoles classes of insecticides the target sites of most interest were the nAChRs, GABA-gated chloride channel and GluCl. However, transcripts encoding various other insecticide target sites were also identified to give a complete overview. All contigs provided in Table 4.7 were manually curated in Geneious to identify open reading frames (ORFs) and allow SNP analysis to be performed.

The GABA-gated chloride channel, VGSC and GluCl target sites had already been assembled using SRA data, discussed in 4.3.1. These three target sites were represented in the transcriptome (Table 4.7) covering the whole of their respective coding sequences. Of these the VGSC AA sequence aligned to *N. vitripennis* VGSC with pairwise similarity of 81.4%. The RDL contig matched that of the previously available RDL sequence in GenBank, whilst the GluCl contig displayed a high (96.3%) AA similarity with a GluCl of *L. striatella*.

There were seven contigs (not including the various duplicates Trinity produced per gene) that represented the nAChR subunits α1-8 and β1. These returned BLAST search hits against a range of insect nAChRs including *P. americana*, *L. migratoria*, *N. vitripennis* and already sequenced *N. lugens* nAChR subunits.

A previous study had published sequences for nAChR subunits α1-4 and β2 (accession no AY378698–AY378700, AY378702, and AY378703) (Liu *et al.*, 2005) but interestingly none of these sequences were the top BLAST hit for nAChR contigs in this transcriptome. This is similar to a previous finding on these sequences, when primers designed from the GenBank *N. lugens* nAChRs could not amplify α1 fragments in *N. lugens* field populations (Puinean, Denholm, *et al.*, 2010). Puinean et al postulate that the taxonomic complexity of *N. lugens*, part of a sibling species group that are morphologically identical and with overlapping host ranges could explain why there is divergence between the sequences. In this analysis, the contig assigned an α1 BLAST hit (c98591), had AA sequence identity of 82.5% with *P. americana* α1, 72.3% with the AY378698 α1 sequence and 98.6% with the partial CDS α1 sequenced by Puinean et al. The same pattern was seen for α2-4 subunits; all show AA similarity to sequences other than those previously sequenced in *N. lugens*. No contig for an α5 subunit was identified in our transcriptome suggesting this subunit may not be expressed in *N. lugens* (there is no gene available in Genbank for *N. lugens*). This correlates with nAChR sequences for *P. americana* (a top BLAST hit species for nAChRs) which does not have an α5 subunit available on GenBank. The remaining subunits α6-8 and β1 were all found to have contigs matching previously sequenced *N. lugens* nAChR subunits. Although, contig c110494 aligned to both a sequenced *N. lugens* α6 subunit (99.2% AA similarity) and to a *P. americana* α7

subunit. Most of the BLAST hits for this contig return α7 sequences. Finally, there was no contig for a β2 subunit found in the transcriptome.

The nAChR subunits were screened for two previously identified mutations that could cause resistance to neonicotinoids. These were R81T from *M. persicae* (Bass, Puinean, *et al.*, 2011) and Y151S in *N. lugens* (Liu *et al.*, 2005). Using the RNA-seq raw reads mapping technique (Chapter II, Section 2.17) the different *N. lugens* populations were screened for these mutations. These mutations were not identified in any of the field strains resistant to imidacloprid (Fig. 4.5), and we conclude that there is no target-site resistance contributing to imidacloprid resistance in our field populations of *N. lugens*.

The two remaining target sites screened for in our transcriptome were the ryanodine receptor (RyR) and the transient receptor potential (TRP) ion channel complex. The contig (c111911) matching RyR aligned to a previously sequenced RyR gene in *N. lugens*. Although insecticides that target RyR are not currently used against *N. lugens*, it is useful to have the target-site sequenced for future reference. The TRP contig showed 59% AA similarity to a TRP gene sequenced in *Plutella xylostella*. This is considerably lower AA similarity than seen for all the other target-sites analysed.

Table 4.7 BLASTx results of GOIs that encode for known insecticide target sites in *N. lugens.*

Gene	Contig	Species	Description	Accession No	% AA Identidy
RDL	c106927	*N. lugens*	RDL	KC841916.1	99
GluCl	c100191	*L. striatella*	GluCl	KU589277.1	96.3
VGSC	c109417	*N. vitripennis*	VGSC transcript variant 1	NM_001134917.1	81.4
RyR	c111911	*N. lugens*	RyR	KJ573636.1	99.4
TRP	c111063	*P. xylostella*	Predicted TRP	XM_011562636.1	59.1
nAChR α1	c98591	*P. americana*	nAChR alpha1	JQ585634.1	82.5
nAChR α2	c105447_g1_i1	*P. americana*	nAChR alpha2	KP725464.1	84.4
nAChR α3	c109754	*L. migratoria*	nAChR3 alpha3	KF873581.1	79.8
nAChR α4	c11527	*N. vitripennis*	Predicted nAChR alpha 4 transcript variant 1	XM_016981584.1	81.8
nAChR α5	-	-	-	-	-
nAChR α6/α7	c110494	*N. lugens*	nAChR alpha6	FJ167396.1	99.2
	c110494	*P. americana*	nAChR alpha7	JX466891.1	84.7
nAChR α8	c99950	*N. lugens*	nAChR alpha8	FJ481979.1	97.8
nAChR β1	c105447_g1_i3	*N. lugens*	nAChR beta1	FJ358493.1	99.8
nAChR β2	-	-	-	-	-

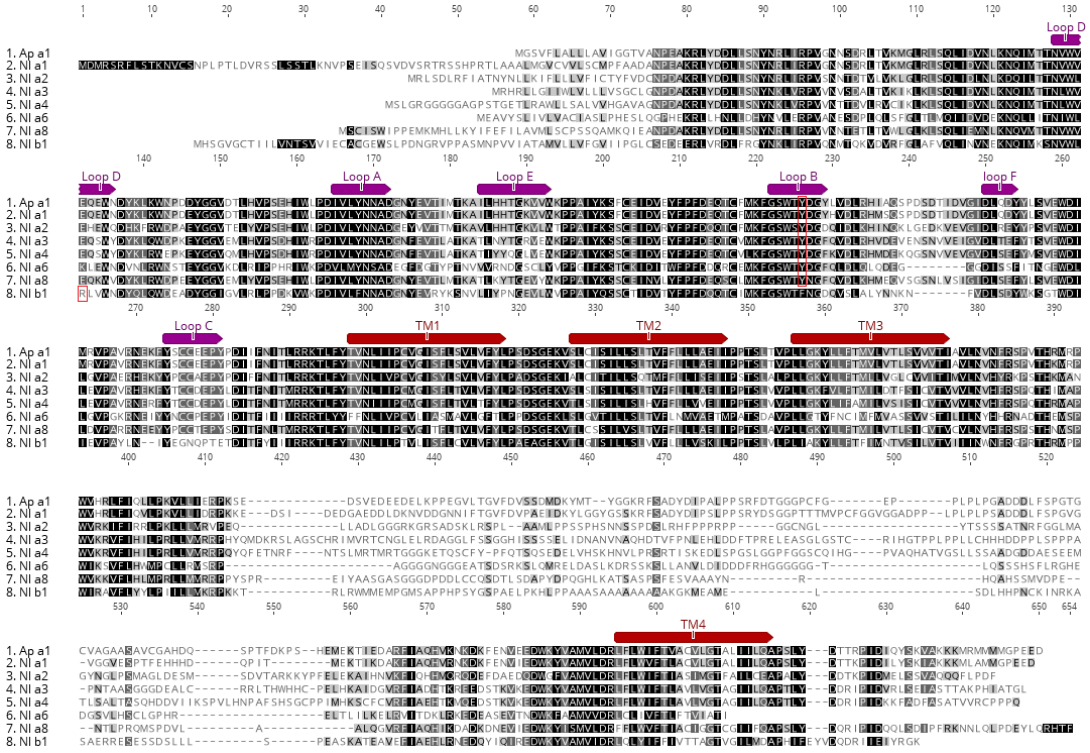


Fig. 4.5 Amino acid sequence alignment of *N. lugens* nAChR subunits found with the transcriptome. The *P. americana* α1 subunit is also displayed. The loop domains are indicated (A-F) by purple bars above the sequence. The transmembrane regions are highlighted by red bars above the sequence. Known mutations involved in neonicotinoid resistance (R81T, Y151S) are boxed in red.

4.4 Conclusions

This study aimed to utilise the available genomic/transcriptomic information to provide a resource for unravelling insecticide resistance in *N. lugens*. This also involved the generation of a *de novo* transcriptome from five different *N. lugens* populations to allow in depth comparisons between insecticide susceptible and resistant populations.

The assembly of three insecticide target sites from publicly available SRA data allowed screening for potential mutations (results in chapter V) before the availability of the *N. lugens* genome, or our own specifically designed RNA-seq

experiment. The creation of our own transcriptome provided a valuable reference for analysing potential genes involved in insecticide resistance. The differential expression analysis of transcripts between the susceptible and resistant strains identified a single strong candidate for further study, a cytochrome P450 gene, *CYP6ER1*, previously implicated in imidacloprid resistance, by showing significant overexpression (24.3-70.2 fold) in all resistant strains compared to Bayer-S. The lack of obvious metabolic enzymes involved in ethiprole resistance suggested that this resistance could be mediated by a target-site rather than metabolic mechanism.

Sequences were assembled for all the known major insecticide target sites. This will provide a valuable resource for screening for potential mutations both for compounds already suffering resistance problems, and any future resistance development. Mapping the RNA-seq raw reads to the genes encoding the target sites, was used as an effective method of SNP analysis. This technique was routinely used throughout this PhD, not just for target site screening but also for searching for allelic variation in metabolic enzymes (chapter VI).

Chapter V Target-site resistance to phenylpyrazoles in *N. lugens*

Much of this chapter (introduction, some of the results and most of the discussion) have been published in *Pesticide Biochemistry and Physiology* (reprints attached in Appendix C) in a paper drafted and written by me.

Garrood, W. T., Zimmer, C. T., Gutbrod, O., Lüke, B., Williamson, M. S., Bass, C., Nauen, R. and Davies, T. G. E. (2017) 'Influence of the RDL A301S mutation in the brown planthopper *Nilaparvata lugens* on the activity of phenylpyrazole insecticides', *Pesticide Biochemistry and Physiology*. Rothamsted Research Ltd, pp. 1–8. doi: 10.1016/j.pestbp.2017.01.007.

Note: Some of the data in this chapter was obtained by employees at Bayer CropScience: Ralf Nauen, Bettina Lüke and Oliver Gutbrod. Figure/table headings indicate their respective contributions.

5.1 Introduction

The phenylpyrazole (fiprole) insecticides, such as ethiprole and fipronil were introduced for *N. lugens* control after resistance to imidacloprid became commonplace (Zhang *et al.*, 2014). Phenylpyrazoles are described as non-competitive blockers of the *gamma*-aminobutyric acid (GABA)-gated chloride channel, a member of the pentameric transmembrane cys-loop ligand-gated ion channel family mediating synapse inhibition in the insect central nervous system (Cole, Nicholson and Casida, 1993; Bloomquist, 2001; Knipple and Soderlund, 2010).

Fiproles are potent inhibitors of GABA-mediated inhibitory nerve transmission and belong to group 2 of the MoA classification scheme of the Insecticide Resistance Action Committee (IRAC), that encompasses GABA-gated chloride channel antagonists (Sparks and Nauen, 2015). This MoA class also includes much older insecticide chemistry, such as the cyclodiene hydrochlorines, which include endosulfan and dieldrin (Casida and Durkin, 2013). Ethiprole is structurally similar to fipronil, only differing in an ethylsulfinyl substituent replacing the trifluoromethylsulfinyl moiety in fipronil (Caboni, Sammelson and Casida, 2003).

Structural change by replacements of alanine 301 in the GABA-gated chloride channel, encoded for by the *Rdl* (*Resistance to dieldrin*) gene, has been linked to high levels of resistance to insecticidal antagonists, in particular cyclodiene organochlorines (ffrench-Constant, 2013). The most common substitution at this position, A301S, was first identified in *D. melanogaster* and shown to cause 4000-fold resistance to dieldrin (ffrench-Constant *et al.*, 1990, 1993). However, the role of this mutation in resistance to the newer fiprole insecticides has been debated (Bass *et al.*, 2004; Remnant *et al.*, 2014). Other mutations at this amino acid residue, situated in the M2 transmembrane domain, have also been associated with fipronil resistance. A 20,000-fold fipronil resistant strain of *Drosophila simulans* exhibited a A301G replacement at this position in combination with a substitution at a second site, T350M in the M3 domain (Le Goff *et al.*, 2005). Functional expression of RDL GABA receptor subunits in *Xenopus* oocytes showed that the A301G mutation has modest effects on fipronil action, while a receptor variant with both of the mutations exhibited higher levels of resistance to fipronil (Le Goff *et al.*, 2005). A third substitution at the A301 position, A301N (A2'N), has been recently associated with

fipronil resistance in two other rice planthopper species, *Sogatella furcifera* (white-backed planthopper) and *Laodelphax striatellus* (small brown planthopper) (Nakao *et al.*, 2010, 2011). In the former species the A301N mutation was identified in association with a R357Q mutation in the cytoplasmic loop between M3 and M4 of the *S. furcifera* RDL GABA receptor subunit with membrane potential assays suggesting the influence of the double mutation on fipronil resistance was more profound than that of the A301N alone (Nakao *et al.*, 2012). This finding parallels that of the earlier work in *Drosophila* suggesting two mutations in the RDL GABA receptor subunit, one at AA residue 301 and one elsewhere act in concert to influence the level of *in vivo* resistance to fipronil (Remnant *et al.*, 2014). However, in contrast to these findings other electrophysiological *in vitro* studies have revealed no significant differences in fipronil antagonist potency between wildtype and A301S RDL GABA receptor variants expressed in *Xenopus* oocytes (Wolff and Wingate, 1998; Lees *et al.*, 2014).

Very recently the A301S mutation was also identified in *N. lugens* and correlated with low levels of resistance to fipronil (5-fold in the presence of enzyme inhibitors and 23-fold without) (Y Zhang *et al.*, 2016). The authors of this study also identified a second substitution in TM2 (R299Q) that in combination with A301S, was associated with much higher levels of resistance in a laboratory selected strain (96-fold with synergists, 237-fold without). Expression of recombinant RDL receptors, demonstrated that the R299Q mutation has a profound effect on the normal functioning of the receptor in response to the endogenous agonist GABA, suggestive of a strong fitness cost. However, the deleterious effects of R299Q was reduced in the presence of the A301S mutation. Surprisingly, the R299Q substitution was

identified at extremely low frequency in field populations of *N. lugens* suggesting this is not the main mechanism of resistance in field populations (Y Zhang *et al.*, 2016).

Due to the evolution of resistance to fipronil in populations of *N. lugens* throughout Asia, and potential issues with the environmental toxicity of this insecticide, most growers subsequently switched to using ethiprole (Bayer CropScience, 2007; Sahaya *et al.*, 2010). Unfortunately, the rapid uptake of this insecticide has led to recent reports of resistance (Garrood *et al.*, 2016). To date, the molecular basis of resistance to this insecticide has not been characterised and the potential role of mutations in the GABA-receptor remain unexplored. Metabolic resistance has been implicated in an ethiprole resistant *N. lugens* field strain from Thailand (Punyawattoe *et al.*, 2013), though the authors also speculated that GABA receptor mutations could play a role in ethiprole resistance. Another study implicated two cytochrome P450s, *CYP4DE1* and *CYP6CW3v2*, in ethiprole resistance in *L. striatellus* (Elzaki, Zhang and Han, 2015).

This chapter discusses the analysis of the phenylpyrazoles target-site, the GABA-gated chloride channel (encoded for by the *Rdl* gene), and its potential role in resistance to these compounds. The work implicates a target-site mutation as a key mechanism behind ethiprole resistance. Further attempts to discover the fipronil resistance mechanism are discussed.

5.2 Material and Methods

5.2.1 *Drosophila* crossing experiments

The wild type strain Canton-S (#1, wild type) and the A301S strain (#35492, Rdl $^{MD\text{-}RR}$) were sourced from the Bloomington *Drosophila* Stock Center at Indiana

University, USA. Strain $RDL^{MD\text{-}RR}$ was homozygous for A301S and highly resistant to ethiprole. Adult virgin females of Canton-S were collected and crossed with adult males of $RDL^{MD\text{-}RR}$. Flies of the F1 generation were then sequenced to check for heterozygosity at the A301S location. All crossings were confirmed to be heterozygous for A301S, and the F1 generation was left to inbreed to produce an F2 population heterogeneous at AA 301. The F2 generation was then screened using a high dose of ethiprole in a bioassay (Chapter II, Section 2.4). Survivors of this bioassay were then screened for the presence of the A301S mutation via sanger sequencing.

5.2.2 gRNA design, plasmid construction and template oligo for CRISPR/Cas9 applications

The two gRNAs, one each for mutations A301S and Q359E, were designed using an online platform flyCRISPR (http://flycrispr.molbio.wisc.edu/targetFinder/) (Gratz *et al.*, 2014). A region spanning ~200 bp either side of A301S (>3L:9148464-9148065) and Q359E (>3L:9145138-9144739) were selected for gRNA design. For A301S the following gRNA (>3L:9148267-9148286 (- strand) CAATGCAACGCCGGCGCGTG) was chosen since it had no off-targets predicted and was located 2 bp away from the nucleotide to be mutated. For Q359E the following gRNA (>3L:9144953-9144972 (- strand) ATACGCCACGGTCGGCTACA) which was predicted to have one off-target event (on the X chromosome), was selected.

gRNA expression plasmids were generated according to the following method. Sense and anti-sense oligos were ordered for the gRNAs designed using flyCRISPR (Appendix A, Table A1). The sense oligos had a GTCG overhang added to the 5' end, whilst the anti-sense oligos had an AAAC overhang added to the 5' end.

The oligos were phosphorylated and annealed to their complement in the following manner. 1 µL of each sense oligo and anti-sense oligo (100 µM), was combined with 1 µL 10X T4 ligation buffer (NEB, Ipswich, MA, USA), 0.5 µL T4 PNK (NEB, Ipswich, MA, USA) and made up to 10 µL with nuclease free water. The reaction was incubated at 37°C for 30 min, then 95°C for 5 min before ramping down to 25°C at 5°C/min. The plasmid chosen to ligate this product into was the pCFD3: U6:3-gRNA plasmid (addgene #49410). This plasmid was linearized prior to the ligation reaction using a restriction enzyme, Bbsl. The ligation reaction consisted of 3 µL of linear plasmid, 1 µL of annealed oligos (1:200 diluted), 1.5 µL 10X T4 ligase buffer (NEB, Ipswich, MA, USA), 0.5 µL T4 ligase and nuclease free water to 15 µL. The reaction was incubated at 22°C for 20 min then placed on ice. Transformation was performed using DH5α™ cells (Thermo Fisher, Waltham, MA, USA). This used 50 µL of DH5α™ cells per reaction, placed in a precooled 2 mL microcentrifuge tube. 5 µL of ligation mix was added to the cells and gently mixed by flicking the tube. This was left to incubate for 30 min on ice, before being heat shocked at 42°C for 25 s. Tubes were placed on ice for 2 min, before addition of 450 µL of pre-warmed LB lysogeny broth. Cells were left to recover for at least 1 h at 37°C with 225 rpm shaking using a shaking incubator (Eppendorf, Hamburg, Germany). 100 µL of each transformation mix was spread on pre-warmed 100 µg/mL AMP (ampicillin) LB plates and incubated over night at 37°C. 8 colonies for each gRNA were picked for colony PCR and a colony record was created, by touching the tips used to pick the colonies onto a fresh AMP LB plate. Colony PCR was performed as previously described (Chapter II, Section 2.13), but using 1 µL of T3 primer and the forward oligo (Appendix A, Table A1). Colonies confirmed to have the correct insert were then processed through plasmid

purification (Chapter II, Section 2.14). After purification plasmids were concentrated to at least 1 µg/µL.

Single stranded oligonucleotides of 110 nt (ssOligo) were synthesized (IDT, Coralville, IA, USA) to be the template for Homology Directed Repair (HDR) after the protein-9 nuclease (Cas9) induced double strand break in the genome, guided by the gRNA plasmids. The template ssOligos (A301S ssOligo and Q359E ssOligo), were both designed to have a SNP that would introduce the respective amino acid substitutions desired (Fig. 5.1). For A301S ssOligo (Fig.5.1A), this would cause a serine (S) to replace the native alanine (A), whilst for Q359E sOligo (Fig. 5.1B) this would introduce a glutamic acid (E) in place of the native glutamine (Q). These ssOligos also contained a SNP just upstream of the mutation sites to prevent Cas9 from re-cleaving the genome after the ssOligo had been incorporated.

Fig. 5.1 Alignment of the 110 nt HDR templates with *Rdl*. A) The first G/T substitution in the gRNA seed sequence (last 12 nt of gRNA), is a designed mismatch to prevent re-cleavage by Cas9. The second G/T substitution causes an amino acid change from alanine (A) to serine (S). B) The first C/G substitution prevents Cas9 re-cleavage, whilst the second C/G introduces an amino acid change from glutamine (Q) to glutamic acid (E).

5.2.3 *Drosophila* embryo injections and screening for CRISPR mediated mutations

Two strains, a DNA ligase 4 deficient strain (#28877, genotype w^{1118} $Lig4^{169}$), and strain expressing endonuclease Cas9 (#51324, genotype w^{1118};

PBac{y[+mDint2]=vas-Cas9}VK00027) were crossed and PCR guided sibling mating rescued a strain designated 'lig4 KO Cas9' (genotype w^{1118} $Lig4^{169}$; Bac{y[+mDint2]=vas-Cas9}VK00027) (Zimmer *et al.*, 2016). This strain was generated by C. Zimmer and maintained in the laboratories at Rothamsted. For the purposes of this study, embryos were collected from this strain and injections were performed as described previously (Chapter II, Section 2.19). There were two injection mixes used: 1) A301S 2) A301S + Q359E. For A301S by itself the mix comprised 0.5 x phosphate buffer (pH 6.8, 0.05mM sodium phosphate, 2.5mM KCL) containing 200 ng μL^{-1} gRNA expression plasmid, 1 µg uL^{-1} template ssOligo and 200 mg L^{-1} fluorescein sodium salt. For the A301S + Q359E mix there was 180 ng μL^{-1} of each gRNA expression plasmid and 800 ng μL^{-1} of both template ssOligos in the 0.5X phosphate buffer with 200 mg L^{-1} fluorescein sodium salt.

Flies that emerged were crossed to a balancer strain, w TM6B tb (#4148, genotype: w^{1118}; Pas^{SC1} gl^{3}/TM6B, gl^{BS1} Tb^{1}) to form generation G0. Flies emerging from this generation were crossed again to the balancer strain to form generation F1. After display of larval activity the fly taken from the G0 generation was collected and screened for the A301S mutation using PCR. When the offspring of the F1 generation emerged as adult flies they were screened with a low dose (1 mg L^{-1}) of dieldrin.

5.2.4 Emergence bioassays (*D. melanogaster*)

Dieldrin (Sigma Aldrich, St. Louis, MO, USA) was added to fly media (concentrations – 5, 1, 0.2, 0.04 and 0.008 mg L^{-1}) at 50^{o}C, with 3 virgin females and

2 males added per vial and allowed to propagate for a 5-day period before being removed. Flies that emerged were scored after 14 days.

5.2.5 Synergist bioassays (*N. lugens*)

Synergist bioassays used the same methodology as for the leaf dip bioassays (Chapter III, Section 3.2.2), with the addition of the following steps. Each insect (*N. lugens*) was treated upon the pronotum with 0.2 µL of 100 mg L^{-1} of an individual synergist in acetone (20 ng $adult^{-1}$). The three synergists used were Piperonyl butoxide (PBO), 1-Aminobenzotriazole (1-ABT) and triflumizole. After synergist application, the insects were transferred to rice stems treated with fipronil. Mortality was assessed at 48 h.

5.3 Results

5.3.1 Sequencing and RNA-Seq read mapping of *N. lugens* GluCl for mutations

cDNA sequencing was conducted for the *N. lugens* glutamate-gated chloride channel (GluCl), using primers (Appendix A, Table A3) designed from the assembled GluCl gene sequence (Chapter IV, Section 4.3.1.1). This was done for the following strains: Bayer-S, Nl33, Nl39, Nl40, Nl55, Nl33-eth, Nl39-eth and Nl55-eth. Sequence analysis of the GluCl in all these strains revealed that there were no putative mutation(s) that could confer resistance compared to the susceptible. This was further validated by RNA-Seq read mapping using the GluCl gene as the reference sequence, which also demonstrated that there were no mutations of interest in resistant strains.

5.3.2 *Rdl* gene structure and expression

The presence of two isoforms for the *Rdl* gene (Chapter IV, Section 4.3.5.2) was further explored using cDNA sequencing, RNA-Seq read mapping and exon calling. Firstly, primers were designed (Appendix A, Table A4) that had a generic forward primer and a specific reverse primer for each isoform. All the strains mentioned previously (Section 5.3.1) were then sequenced using these primers, revealing that both *Rdl* isoforms were being expressed across the populations.

The *N. lugens* genome assembly was searched for scaffolds containing the *Rdl* gene sequence. This returned two scaffolds, 387_19 and 387_20 (accession no AOSB01071559-60), that contained most of the CDS sequence for both *Rdl* isoforms. Searching the genome assembly using specific nucleotide sequences for each isoform (from where the sequences diverge), highlighted that the two isoforms were alternate splice forms of the final exon: 9A and 9B (Fig. 5.2) located on scaffold 387_20.

Fig. 5.2 The exon organisation of exon 9A and exon 9B of the *N. lugens Rdl* gene on genome scaffold 387_20

Attempts to quantify the level of expression of these two exons using qRT-PCR were unsuccessful due to the inability to design sufficiently distinct primer pairs. However, using the RNA-Seq raw reads to map back to the exons demonstrated that one was clearly more highly expressed than the other (Table 5.1). Across all the

strains the 9B exon was the preferred exon expressed, rather than that represented in the original GenBank sequence (accession no. KC841916.1).

Table 5.1 Raw reads aligning to the *Rdl* alternate exon.

	No. reads	
	9AExon	9B Exon
Bayer-S	15	54
Nl33	16	83
Nl55	17	43
Nl33-eth	24	142
Nl55-eth	10	43
Total	82	365

Since this*Rdl* isoform (9B), was the dominant form seen across all the strains, this was the isoform employed in the subsequent experiments described in this chapter.

5.3.3 Screening *N. lugens Rdl* for mutations

The cDNA sequencing and RNA-Seq read mapping of N. *lugens Rdl* revealed two non-synonymous mutations in the fiprole resistant strains compared to the susceptible strain. These were A301S and Q359E (Fig. 5.3). The A301S mutation was seen intermittently across all the field populations and in the ethiprole selected strains (primers, Appendix A, Table A4). However, Q359E, was only seen in strains from India (Nl55, Nl56 and Nl55-eth) and not strains from Vietnam (Nl33, Nl33-eth, Nl39 and Nl39-eth). No other mutations (R299Q, A301G/N, T350M and R357Q) that had been previously linked with resistance to phenylpyrazoles were seen across the highly fiprole resistant populations studied here (primers, Appendix A, Table A4).

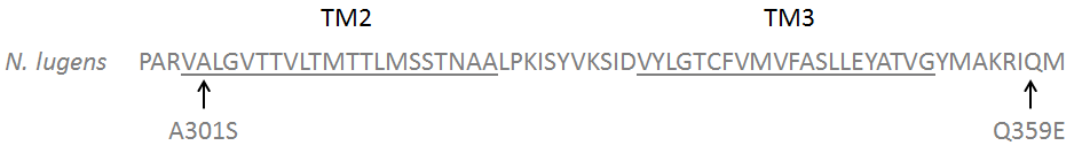


Fig. 5.3 Amino acid sequence of TM2 and TM3 (TM regions underlined) from *N. lugens* RDL. The alanine and glutamine residues that are mutated in fiprole resistant strains are highlighted.

5.3.4 Genotyping A301S and Q359E via Sanger Sequencing

The cDNA sequencing performed to screen the *Rdl* for mutations was a combination of pooled insects and cloned PCR products. Therefore, to explore the levels of these two mutations in the *N. lugens* populations, sequencing was done on single insects to allow individual genotyping. Since previous sequencing efforts had shown that Q359E was only present in Indian strains this genotyping focused on Bayer-S, Nl55 and Nl55-eth.

All strains were analysed for the presence of the A301S mutation by Sanger sequencing of an amplified 257 bp sequence from genomic DNA. Genotyping of A301S in Bayer-S confirmed the wild type A301 genotype (Table 5.2). In comparison, Nl55 displayed a mix of genotypes, with only 12.5% of insects homozygous for 301S and 32.5% homozygous for the wildtype genotype. 100% of insects analysed from Nl55-eth carried the A301S mutation in the homozygous form. The novel mutation, Q359E, was also genotyped for Nl55 and Nl55-eth. Nl55 displayed 7% of individuals homozygous for the 359E mutation, with 57% of insects homozygous for the wildtype genotype. However, 87% of individuals were homozygous for the Q359E mutation in Nl55-eth, while the remaining 13% were heterozygous. Since the A301S mutation

reached fixation in Nl55-eth it can be concluded that there are two A301S alleles present in that strain, one with and one without the Q359E mutation. The potential that these two mutations could be linked (linkage disequilibrium) is discussed later as an explanation as to how the Q359E mutation and the A301S come to be seen in tandem.

Table 5.2 Genotypes via Sanger sequencing of *N. lugens* strains for A301S and Q359E.

Population	A301S genotype (%)			Q359E genotype (%)		
	RR	SR	SS	RR	SR	SS
Bayer-S	0	0	100	0	0	100
Nl55	12.5	55	32.5	7.14	35.71	57.14
Nl55-eth	100	0	0	86.84	13.16	0

(Nl55 A301S N=40, Nl55-eth A301S N=40, Nl55 Q359E N=28 and Nl55-eth Q359E N=38)

5.3.5 SNP calling of A301S and Q359E via RNA-Seq

For the strains that had been sequenced using Illumina (Chapter IV, Section 4.2.2), RNA-Seq reads were mapped against the *N. lugens Rdl* nucleotide sequence to observe any non-synonymous mutations, of which there were two: A301S and Q359E. Bayer-S displayed 100% of reads containing the wild type genotype at AA residue 301 (Table 5.3). Nl33 and Nl55 exhibited 85% and 80% of reads with the wild type genotype respectively. For the Nl33-eth and Nl55-eth populations, 100% of reads contained the A301S mutation. In agreement with the Sanger sequencing, SNP calling of RNA-Seq data showed that the Q359E mutation was only found in Nl55 and Nl55-eth (Table 5.3), with 96% of Nl55-eth reads containing the Q359E mutation, compared to 27% for Nl55.

Table 5.3 SNP calling via RNA-Seq of *N. lugens* strains for A301S and Q359E.

		Nl33		Nl33-eth		Nl55		Nl55-eth	
		No. reads	%	No. reads	%	No. reads	%	No. reads	%
A301S	Total reads	20	-	34	-	10	-	18	-
	G (WT)	17	85	0	0	8	80	0	0
	T (Mut)	3	15	34	100	2	20	18	100
Q359E	Total reads	25	-	30	-	11	-	23	-
	C (WT)	25	100	30	100	8	72.73	1	4.35
	G (Mut)	0	0	0	0	3	27.27	22	95.65

5.3.6 Functional validation of A301S and Q359E

To functionally validate the mutations discovered in *Rdl* numerous experiments were performed. These included electrophysiological studies, *D. melanogaster* insecticide mortality bioassays and crossings, trikinetic measurements and attempted CRISPR mediated introduction of the mutations into *D. melanogaster*. Of these it was decided that the *Drosophila* experiments would be conducted by myself, whilst the electrophysiology studies would be organised and performed by Bayer CropScience personnel. The electrophysiological studies are not presented in the results section but are discussed later.

5.3.6.1 *D. melanogaster* fiprole bioassays

The $RDL^{MD\text{-}RR}$ (carrying *Rdl* A301S) *D. melanogaster* strain displayed high levels of resistance to ethiprole with a resistance ratio >4000 fold based on the LC_{50} when compared with the wildtype *D. melanogaster* strain, Canton-S (Table 5.4). Against fipronil the $RDL^{MD\text{-}RR}$ strain had a resistance ratio of only 6.9-fold.

The $RDL^{MD\text{-}RR}$ strain was sequenced across the A301S region to confirm this mutation and to check that there were no other non-synonymous mutations in this region. A further region, just after TM3, was also sequenced (primers, Appendix A,

Table A4) to ensure that mutations (T350S and M360I) evaluated in a previous study (Remnant *et al.*, 2014) were not present in this strain. No other mutations were found, confirming that this strain only contained the A301S mutation.

Table 5.4 Log-dose probit mortality data for fiproles against *D. melanogaster* strains.

Compound	Strain	LC_{50} [mg L^{-1}]	95% CL	LC_{95} [mg L^{-1}]	95% CL	Slope (± SD)	Resistance Ratio LC_{50}	Resistance Ratio LC_{95}
Ethiprole	Canton-S	5.73	4.77-6.77	22.39	17.14-33.08	2.777 ± 0.238	1	1
	RDL^{MD-RR}	>25000	-	>25000	-	-	>4300	>1100
Fipronil	Canton-S	1.27	0.77-1.85	9.04	5.29-25.55	1.931 ± 0.333	1	1
	RDL^{MD-RR}	8.82	5.34-13.7	62.36	33.11-238.1	1.936 ± 0.363	6.9	6.9

5.3.6.2 Crossing experiments for Canton-S and RDL^{MD-RR}

Approximately 200 flies of the F2 generation of the Canton-S x RDL^{MD-RR} strain were placed on agar vials spread with 500 mg L^{-1} ethiprole. From this bioassay, there were approximately 50 survivors (25%). Survivors from this insecticide screening were collected and 5 flies underwent DNA extraction. PCR sequencing of the DNA spanning the A301S region showed that the insecticide screening survivors were all homozygous for the A301S mutation (Fig. 5.4).

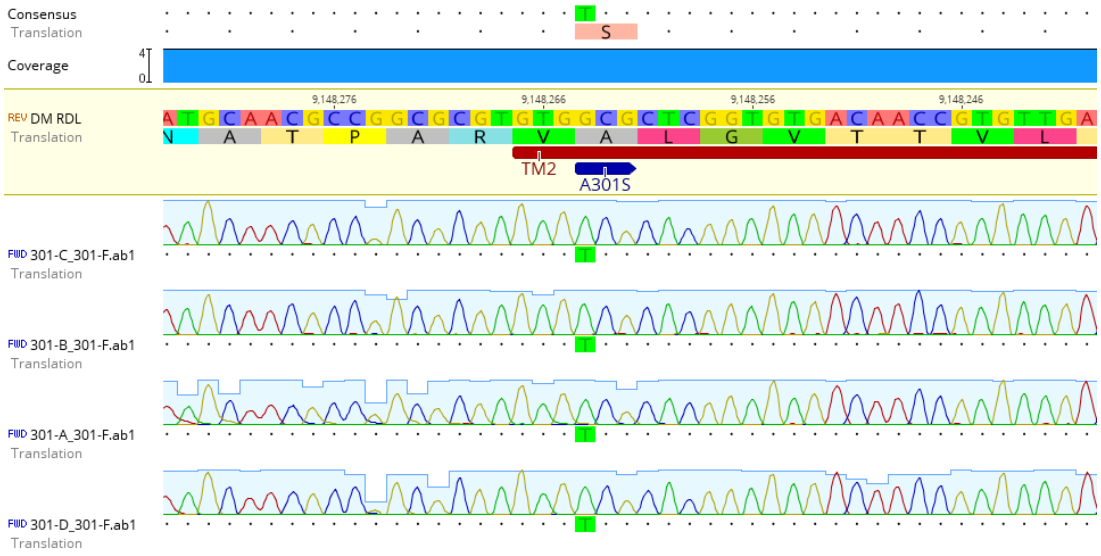


Fig. 5.4 Genotyping for A301S in ethiprole screened survivors. Sequencing chromatograms show that all survivors are homozygous for the A301S mutation.

5.3.6.3 Locomotor activity of ethiprole treated *Drosophila* strains

Whilst visiting the Batterham Lab at the University of Melbourne, I had the chance to attempt various experiments to study the effects of insecticide resistance using *D. melanogaster* techniques that were unavailable at Rothamsted Research. The first of these was monitoring locomotor activity in *D. melanogaster* (Chapter II, Section 2.18) strains in the presence of insecticide.

Two strains, Canton-S and RDL^{MD-RR}, were analysed using this trikinetics system. The effect on activity of these two strains, in the presence of ethiprole, was very different (Fig. 5.5). The Canton-S strain, at 500 mg L^{-1} ethiprole, displays normal activity for the first 10 h before the activity rapidly decreases. There is a slight recovery, but no further movement is recorded after 23 h (Fig. 5.5A). In comparison, the RDL^{MD-RR} strain always shows movement. It is appreciably lower than the control for the first 15 h, however it shows peaks at similar timepoints as the control in this period (Fig. 5.5C). After this period the strain recovers to levels above that of the control.

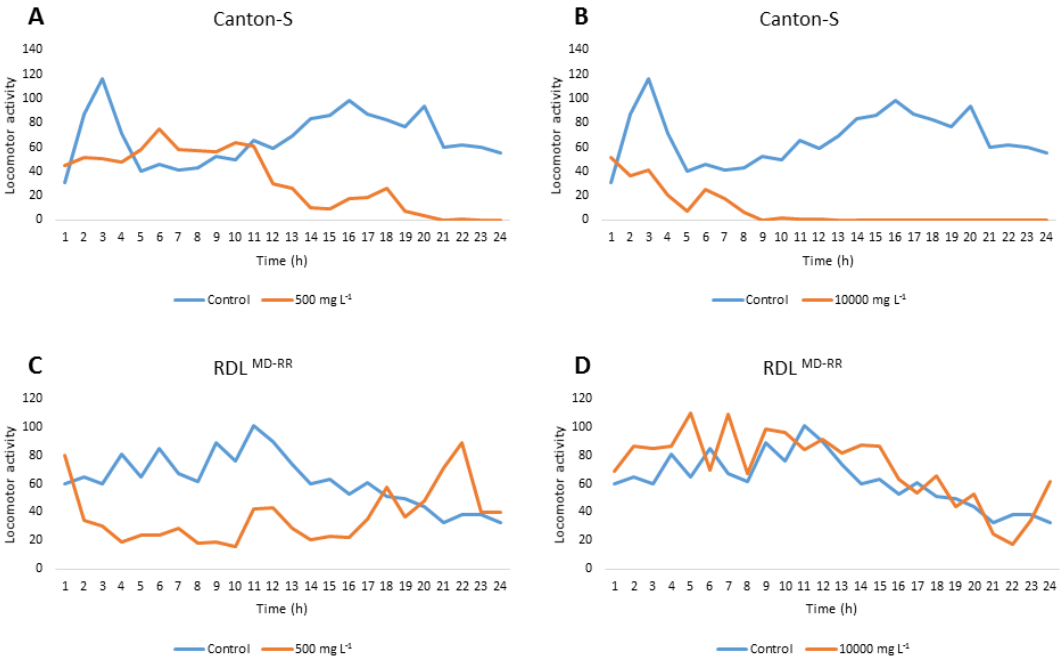


Fig. 5.5 Locomotor activity of Canton-S and RDL^{MD-RR}. A) Canton-S treated with 500 mg L^{-1} ethiprole. B) Canton-S treated with 10000 mg L^{-1} ethiprole. C) RDL^{MD-RR} treated with 500 mg L^{-1} ethiprole. D) RDL^{MD-RR} treated with 10000 mg L^{-1} ethiprole. Time axis displays the number of hours the flies have been on the ethiprole coated food. Locomotor activity displayed is the average movement recorded for each of the 5 min sections within a given hour.

At 10000 mg L^{-1} ethiprole the response of Canton-S is even more pronounced than at 500 mg L^{-1} with a swift knockdown in movement, followed by a brief increase

in movement before movement gradually ceases, with no significant movement recorded after 9 h (Fig. 5.5B). For $RDL^{MD\text{-}RR}$ the flies movement is closer to that of the control when compared at the 10000 mg/L^{-1} than 500 mg/L^{-1} ethiprole, and why this is the case is not clear. Again there is no time point where no movement is recorded (Fig. 5.5D). Also at the end of the experiment (approximately 2 days, data not shown here) there is still significant movement occuring in the $RDL^{MD\text{-}RR}$ strain despite the very high levels of insecticide present.

5.3.6.4 CRISPR mediated A301S and Q359E replacement in *D. melanogaster*

Approximately 300 embryos were injected with each injection mixture (A301S alone/A301S + Q359E combined). The flies that emerged from the injected embryos (~14%) were then crossed to the chromosome 3 balancer stock (w TM6B tb) and maintained on normal fly media. This is designated the G0 generation.

Exploratory experiments were done to determine whether insecticide screening could be performed on this first fly cross, in the manner previously described (Zimmer *et al.*, 2016). However, attempts to find a dieldrin dose that could be used to supplement the food which let adults survive, but killing susceptible larvae, were unsuccessful. The lowest tested dose that the Lig4 KO Cas9 adult flies could survive on was 0.2 mg L^{-1} (Table 5.5). But at this dose susceptible larvae could survive and emerge as adults (Fig. 5.6), so no discriminatory screening was being implemented. Higher dieldrin doses led to full mortality of the Lig4 KO Cas9 adult flies. It was therefore decided not to use insecticide supplemented media as a screening agent for these fly crosses.

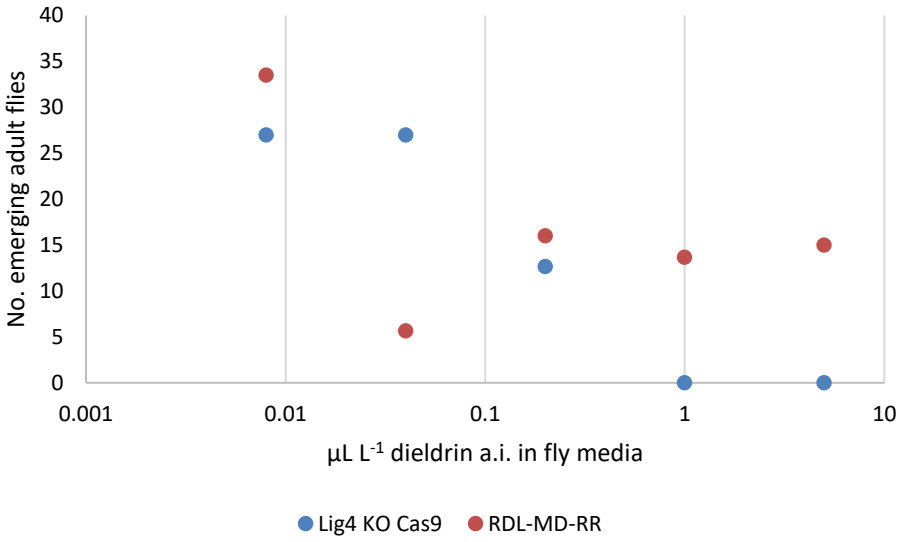


Fig. 5.6 Concentration-response relationship for an emergence bioassay with Lig4 KO Cas9 and RDL-MD-RR strains. A.i. = active ingredient.

For the G0 crosses involving flies injected with the A301S + Q359E mix, 40 adult flies emerging from the original cross were again crossed to the same balancer strain to form the F1 generation These new crosses were left to propagate until larvae activity was detected then the relevant fly was collected. The flies that were collected for PCR analysis were those that had been taken from the G0 generation. PCR was run and sequenced to assess introduction of mutations A301S and Q359E (primers, Appendix A, Table A4). Sequencing of 42 flies revealed that none contained either of the mutations.

Table 5.5 Mortalities (%) of fly strains to dieldrin after 72 h.

Concentration (mg L^{-1})	Lig4 KO Cas9
5	100
1	100
0.2	6.6
0.04	6.6
0.008	0

The same result was obtained for the flies involved in the A301S only cross. PCR and sequencing of flies emerging from the original cross confirmed no introduction of the A301S mutation. To double check this finding the flies from the F1 cross were inbred for a further generation, to allow an insecticide screen to be performed. However, no flies from this generation survived a screen with a low dose of dieldrin. A few flies from this bioassay were collected for PCR and sequencing which again demonstrated no incorporation of the A301S mutation.

5.3.7 Synergist bioassays

5.3.7.1 PBO + fipronil

Synergistic bioassays were conducted with PBO on the highly fipronil resistant populations Nl33-eth, Nl39-eth and Nl55-eth to assess whether P450 monooxygenases (and/or esterases) could potentially be contributing to the resistance phenotype observed. The resistance of these strains to fipronil in the absence of PBO was displayed previously (Chapter III, Table 3.8), with LC_{50}s over 1000 mg L^{-1}. The fipronil mortality of all PBO treated populations was under 25% at a fipronil concentration of 100 mg L^{-1} and 500 mg L^{-1} (Table 5.6). This indicated that most of the individuals of all three strains are unaffected by the application of PBO prior to exposure to fipronil.

Table 5.6 Mortalities (%) of ethiprole selected populations to fipronil after application of 0.2 µL of 100 mg L^{-1} (20 ng $adult^{-1}$) PBO.

Strain	100 mg L^{-1}	500 mg L^{-1}
Nl33-eth	0.2	0.16
Nl39-eth	0	0.082
Nl55-eth	0.1	0.24

5.3.7.2 1-ABT and triflumizole + fipronil

Further synergistic bioassays were conducted with two alternative compounds: 1-ABT and triflumizole. This was to assess whether other P450 monooxygenase inhibitors could have any impact on fipronil susceptibility. Nl39-eth fipronil mortality was below 15% after the application of 500 mg L^{-1} fipronil following 1-ABT treatment (Table 5.7). When triflumizole was applied the mortality at 500 mg L^{-1} fipronil was still below 30%. This is broadly in line with the results seen when the fipronil resistant populations were treated with PBO before fipronil exposure.

Table 5.7 Mortalities (%) of Nl39-eth to fipronil after application of different synergists.

	Nl39-eth	
Synergist	100 mg L^{-1}	500 mg L^{-1}
1-ABT	0.05	0.11
Triflumizole	0.23	0.29

5.3.8 Fipronil sulfone bioassays

A primary metabolite of fipronil, fipronil sulfone, was also tested against the fipronil resistant populations of *N. lugens*. The Nl55 strain had very low resistance to fipronil (RR 3), compared to the ethiprole selected strains (RR >860), and a similar trend is apparent for fipronil sulfone. Only a few dose concentrations were tested for fipronil sulfone, so an accurate LD_{50} was not calculated for the strains. However, at 20 mg L^{-1} fipronil sulfone Nl55 mortality was 80% (Table 5.8) whilst for the Nl33-eth strain only 20% mortality was recorded at the same dose. At 100 mg L^{-1} all the Nl55 brown planthoppers were dead whilst there was still considerable survivorship for Nl39-eth (63%). It is not possible to compare mortalities at certain dose/concentrations between fipronil and fipronil sulfone bioassays due to the different bioassay methodology used to assess resistance to these compounds.

Table 5.8 Mortalities (%) of *N. lugens* strains to fipronil sulfone.

	Fipronil Sulfone		
Strain	20 mg L^{-1}	100 mg l^{-1}	500 mg L^{-1}
Nl55	80.9	100	100
Nl33-eth	21.5	69.9	94.5
Nl39-eth	nt	37.5	70.7

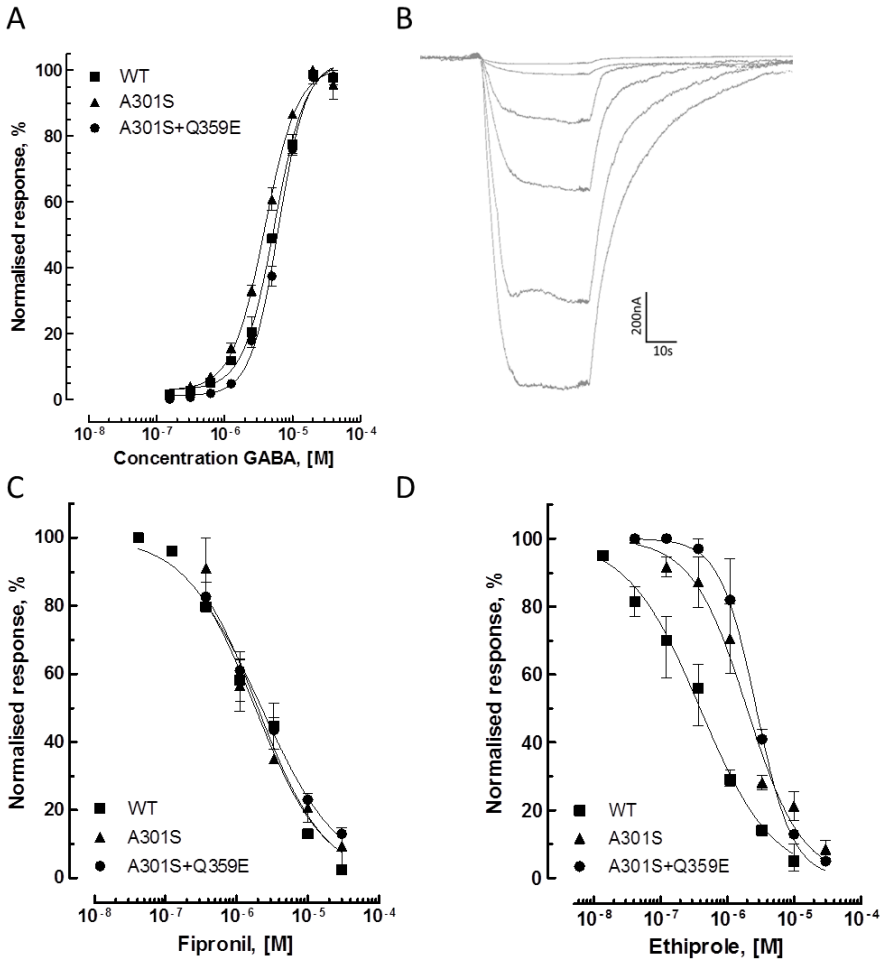
5.4 Discussion

To date, the molecular basis of ethiprole resistance in *N. lugens* has remained unclear. A previous study linked esterase activity, and to a lesser extent P450s activity, to ethiprole resistance in *N. lugens* in central Thailand, based on the separate application of PBO, triphenyl phosphate and diethyl maleate as synergists prior to ethiprole exposure (Punyawattoe *et al.*, 2013) . However, to date, no mutation(s) in the non-competitive antagonist binding site of the RDL channel has been implicated in resistance to ethiprole. In the case of fipronil resistance, a potential novel mechanism of resistance was very recently implicated in a laboratory selected strain of *N. lugens* (see introduction to Chapter V), but was not observed at sufficient frequency to cause resistance in field populations (Y Zhang *et al.*, 2016) .

In this chapter, two mutations in *Rdl* associated with phenylpyrazole resistance in two field strains were identified. Field strains, Nl33 (Vietnam) and Nl55 (India) exhibited high levels of resistance to ethiprole, despite a long period of non-selection (27 and 18 generations respectively), as discussed in Chapter III. When these strains were exposed to continuous ethiprole selection, their resistance markedly increased compared to the non-selected populations. Two mutations were identified in these strains; the first was the previously reported A301S mutation (Y Zhang *et al.*, 2016), which was observed at low frequency in both parental field strains but rapidly rose in frequency and became fixed under ethiprole-selection. A

further, novel mutation was identified, Q359E, in one of the strains that also increased in frequency under selection. Subsequent functional analysis of the role of these mutations in resistance to fipronil and ethiprole, provided several lines of evidence to support a causal role of the A301S mutation in resistance to ethiprole, but not fipronil.

Firstly, *in vivo* evidence of the role of this mutation in ethiprole resistance was provided by insecticide bioassays of a *D. melanogaster* $RDL^{MD\text{-}RR}$ line with the same mutation, which exhibited 4000-fold resistance to ethiprole in comparison to a strain (Canton-S) without the mutation. Locomotor activity assays also revealed a remarkable difference in susceptibility to ethiprole between these two *D. melanogaster* strains, with the strain carrying A301S being broadly unaffected by ethiprole exposure. A simple crossing experiment between $RDL^{MD\text{-}RR}$ and Canton-S, followed by selection on a high concentration of ethiprole, demonstrated that all survivors were homozygous for A301S. Further conclusive evidence was provided through expression of recombinant wild-type and A301S RDL receptors in *Xenopus* oocytes followed by electrophysiological assays (Fig. 5.7, conducted by Bayer CropScience). These showed that the presence of the A301S mutation reduces the sensitivity of the receptor to ethiprole 8-10-fold compared to wild-type, providing strong evidence of a role *in vitro*.



Source: (Garrood *et al.*, 2017)

Fig. 5.7 Effect of GABA and fiprole antagonists on GABA-induced currents in *N. lugens* RDL receptors functionally expressed in *Xenopus* oocytes. (A) GABA concentration-response curves on wildtype (WT) and mutated RDL variants carrying an A301S and A301S+Q359E amino acid substitution, respectively. Data are mean values ± SEM (n=3); (B) Typical example of electrophysiological oocyte recordings showing the concentration-dependent action of GABA (10, 5, 2.5, 1.25, 0.625, 0.313 and 0.156 µM) on functionally expressed receptors (Rdl A301S). (C, D) Antagonist concentration-response curves for fipronil and ethiprole on the three different RDL variants. The responses were normalised relative to the currents induced by 5 µM GABA for each receptor variant. Data are mean values ± SEM of 3-5 independent recordings. Data obtained by R. Nauen and B. Lüke (Bayer CropScience).

In contrast to the findings with ethiprole very limited evidence was seen for a causal role of the A301S mutation in resistance to fipronil. A low level of resistance to fipronil was seen in the *D. melanogaster* line RDL$^{MD\text{-}RR}$ with the A301S mutation

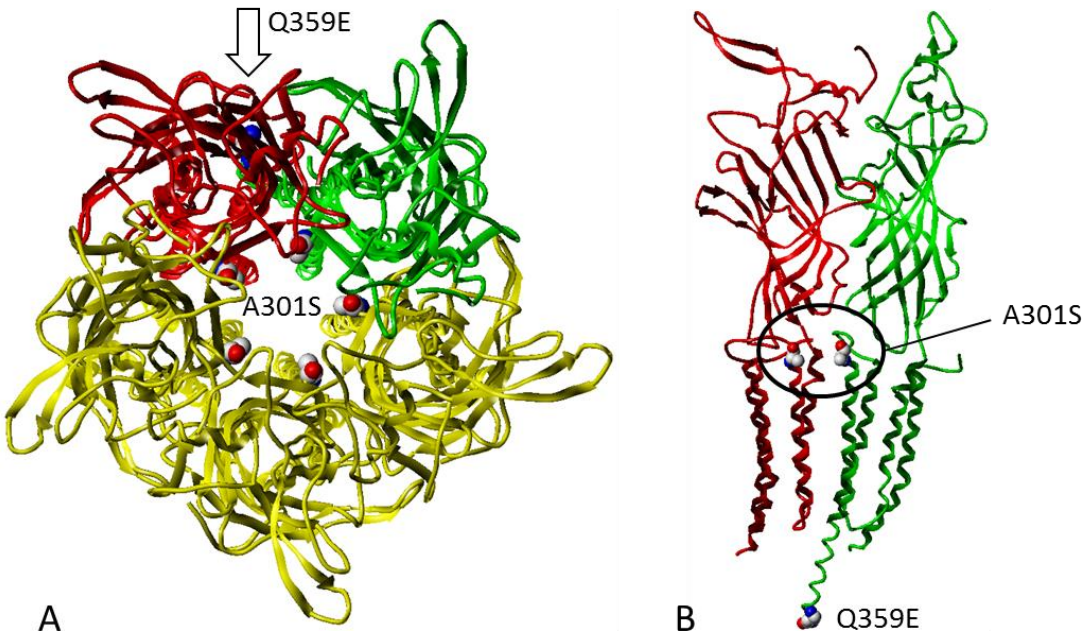
(around 7-fold compared to Canton-S). This result is similar to the 13.8-fold resistance reported by Remnant *et al.*, 2014 on the same *D. melanogaster* strain. A different strain of *D. melanogaster* (*mel-ser*) carrying the same A301S mutation was previously reported to show 73-fold resistance to fipronil (Cole, Roush and Casida, 1995), however, such high levels of resistance to fipronil were not apparent in this present study or that carried out previously by Remnant et al 2014. This conclusion is reinforced by the Bayer CropScience electrophysiological assays where the recombinant A301S RDL receptor showed no significant shift in sensitivity to fipronil, with a response broadly similar to that of the wild type receptor.

As detailed above a second mutation, Q359E, was also observed at low frequency in the Nl55 strain sourced from India but was selected for at high frequency upon ethiprole selection in the laboratory. All insects identified as having the Q359E substitution carried it in combination with A301S. Since this mutation is never seen in isolation in the selected *N. lugens* population, it was decided to focus the analysis on the double mutant (A301S + Q359E) via electrophysiology to assess the effect of Q359E in tandem with A301S.

The data provided by Bayer CropScience suggests that in contrast to A301S, the Q359E mutation plays no direct role in resistance to either ethiprole or fipronil with A301S/Q359E receptors displaying the same level of sensitivity to both compounds as the A301S modified receptor. Attempts to analyse Q359E *in vivo* by introducing the mutation into the *D. melanogaster Rdl* gene were unsuccessful. The most likely explanation for the failed integration of the mutation was the experimental strategy of trying to insert the A301S and Q359E mutations

simultaneously (within the same injection round). Within the *D. melanogaster* genome these two sites are located approximately 3.3 kb apart, and it was presumed that this was a sufficient distance to introduce two separate cut sites for HDR. However, PCR analysis revealed that these two cut points were close enough to cause the entire 3.3 kb region to be excised, and explains why no flies with the desired mutations were obtained.

A model of the *N. lugens* RDL GABA-R homo-pentamer (provided by Bayer CropScience) places the Q359E mutation at least >40 Å away from the key A301S residue which could be a potential reason for its lack of direct impact (Fig. 5.8). However, a previous study using the Drosophila Genetic Reference Panel (DGRP) lines identified three fipronil resistant strains (A301S + T350S, A301S + T360I and A/S301 + M/I360) demonstrating the ability of multiple mutations in the *Rdl* to cause fipronil insensitivity (Remnant *et al.*, 2014).Duplication at the *Rdl* locus has been described, and demonstrated the ability to accrue resistance mutations but maintain wildtype functionality in this insecticide target site (Remnant *et al.*, 2013).



Source: (Garrood *et al.*, 2017)

Fig. 5.8 A) Top-view of the RDL GABA-R homo-pentamer (*Nl* RDL homology model based on 3RHW) showing three subunits in yellow, one in green and red, respectively. A301S is located in the middle of the M2 transmembrane helices forming the channel pore. Q359E is located intracellularly at the end of helix M3 outside the pore region (indicated by an arrow). B) Side-view showing two of the RDL subunits and the location of A301S in transmembrane pore helix M2, whereas Q359E is located more than 40 Å from this residue (the helical structure of the domain is proposed as amino acid positions 337-428 are missing in the modelling template 3RHW). Model generated by O. Gutbrod (Bayer CropScience).

In the light of these results there are two possible explanations for the increase in frequency of the Q359E mutation under ethiprole selection. Firstly, it is a random polymorphism that, because of a close proximity to A301S, has hitchhiked to high frequency due to the physical linkage (linkage disequilibrium) of the two mutations and the adaptive advantage of A301S. Secondly, this mutation, while not directly contributing to ethiprole resistance, may have a fitness benefit, to *N. lugens* individuals that carry this mutation in combination with A301S. For example, the Q359E mutation might act as a compensatory mutation for A301S as has been

recently claimed for the R299Q substitution (see Introduction)..This seems unlikely since, A301S has been shown to persist in other insect species at high frequency in the absence of insecticide selection (Thompson, Steichen and ffrench-Constant, 1993), suggesting it may have a minimal fitness penalty. Also, recombinant receptors with A301S alone and A301S+Q359E showed the same affinity for the native ligand GABA.

The A301S mutation was one of the first target-site resistance mutations to be described in insects and has since appeared in a wide array of different insect species (ffrench-Constant, R. H. Anthony *et al.*, 2000). Originally described as the primary mechanisms of resistance to cyclodienes, it has also been linked with low level cross-resistance to fipronil in *Ctenocephalides felis* and *D. melanogaster* (Bass *et al.*, 2004; Remnant *et al.*, 2014). The effect of A301S in relation to cyclodiene resistance is two-fold, it reduces insecticide binding and destabilises the antagonist favoured structure of the RDL channel (Zhang, ffrench-Constant and Jackson, 1994). Surprisingly, this mutation has never been previously implicated in ethiprole resistance. Fipronil and ethiprole are structurally very similar (Fig. 1.6) and so it is also surprising that the A301S mutation can provide such effective resistance against ethiprole, but not to the same extent against fipronil.

The extremely high resistance levels seen in *N. lugens* strains selected with ethiprole, cannot be completely explained by the *Rdl* A301S mutation. The difference between wild-type and A301S RDL receptor constructs in the voltage clamp recordings, was not enough to be wholly responsible for the resistance described in Chapter III. Therefore, there must be another mechanism of resistance capable of

causing resistance to ethiprole within the *N. lugens* populations tested here. One can hypothesise that the unknown fipronil resistance mechanism (discussed later) could cause cross resistance to ethiprole, and therefore explain the very high levels of resistance in these *N. lugens* strains.

Zhang et al.'s recent study on R299Q and A301S mutations in the RDL GABA receptor and their correlation with fipronil resistance (Y Zhang *et al.*, 2016), has similarities with the work discussed in this chapter. The finding of a novel mutation existing only in tandem with the A301S mutation is a key finding. However, the new mutation they describe (R299Q) appears to increase resistance to fipronil when combined with A301S, further than A301S by itself. Although Q359E is a novel mutation, it does not have the same direct impact as R299Q. Similar to results reported by Zhang et al. the RDL GABA receptor mutations analysed in this current study are not the main mechanism of resistance to fipronil in the *N. lugens* populations tested.

The lack of impact of either of the RDL GABA receptor mutations against fipronil led to tests with multiple P450 inhibitors (PBO, 1-ABT and triflumizole) to explore if this enzyme system is involved in conferring resistance to this compound. In this regard, recent research has used the same approach to implicate metabolic mechanisms in resistance to fipronil in *N. lugens* (Y Zhang *et al.*, 2016). In this current study the application of PBO had no noticeable impact on the fipronil resistance of the resistant populations, Nl33-eth and Nl55-eth suggesting P450s and/or esterases are either not involved in resistance or play a minor role. Further applications with 1-ABT and triflumizole also had negligible impact on fipronil resistance in the resistant

strain, Nl39-eth, strengthening the belief that P450s are not a major driver of fipronil resistance. However, Zhang et al. applied a mixture of synergists (PBO, triphenyl phosphate and diethyl maleate), so it is possible that other enzyme systems that are inhibited by triphenyl phosphate and/or diethyl maleate are involved in resistance such as glutathione S-transferases (Y Zhang *et al.*, 2016).

Fipronil resistant *N. lugens* strains were also resistant to a primary metabolite of fipronil, fipronil sulfone. Fipronil sulfone also targets the GABA-gated chloride channel (Hainzl, Cole and Casida, 1998) and it has been debated whether conversion of fipronil to fipronil sulfone is a detoxification process (Zhao *et al.*, 2005). Oxidation of fipronil by P450s to fipronil sulfone, has been shown as a detoxifying process, by studies showing an increase in fipronil toxicity when pre-treatment with PBO occurs (Hainzl and Casida, 1996; Caboni, Sammelson and Casida, 2003). Since the fipronil resistant *N. lugens* strains are also highly resistant to fipronil sulfone compared to a unselected field strain (Nl55), there is likely a common resistance mechanism for both compounds. Resistance to fipronil sulfone demonstrates that resistance to fipronil is not due to downregulation of P450s involved in oxidation of fipronil.

5.5 Conclusions

Two mutations (A301S and Q359E) were identified in the *Rdl* gene of *N. lugens* and assessed for their potential role in resistance to fiproles. The results obtained indicate that the common A301S mutation confers resistance to ethiprole, a widely-used insecticide for the control of brown planthopper. However, neither this mutation nor the novel mutation Q359E causes significant resistance to fipronil based on the *in vitro* and *in vivo* studies discussed here. Upregulation of P450s does

not appear to play a role in fipronil resistance. Since the A301S mutation cannot fully explain the resistance to ethiprole, it is hypothesised that uncharacterised metabolic resistance within the *N. lugens* populations studied here could also provide a substantial degree of cross-resistance to ethiprole.

Chapter VI Metabolic resistance to neonicotinoids and phenylpyrazoles insecticides in *N. lugens*

Some of the results presented in this chapter have been published in peer reviewed journals.

Garrood, W. T., Zimmer, C. T., Gorman, K. J., Nauen, R., Bass, C. and Davies, T. G. E. (2016). 'Field-evolved resistance to imidacloprid and ethiprole in populations of brown planthopper *Nilaparvata lugens* collected from across South and East Asia', *Pest Management Science,* 72**,** 140-9.

Christoph T. Zimmer[#], **William T. Garrood**[#], Kumar Saurabh Singh, Emma Randall, Bettina Lueke , Oliver Gutbrod , Svend Matthiesen , Maxie Kohler , Ralf Nauen , T.G. Emyr Davies and Chris Bass (2018) 'Neofunctionalization of duplicated P450 genes drives the evolution of insecticide resistance in the brown planthopper', *Current Biology*, 28, 268-274.

[#]Contributed equally

6.1 Introduction

A variety of mechanisms have been proposed to underlie the widespread resistance to neonicotinoids, especially imidacloprid, in *N. lugens* across Asia. These have included both target-site and metabolic mechanisms. The first mechanism demonstrated to decrease imidacloprid susceptibility, was a target-site mutation. This study was performed before the appearance of widespread control failure in the field, and used a laboratory-selected strain of *N. lugens*. A single point mutation was identified (Y151S) at a conserved position in two nAChR subunits, Nlα 1 and Nlα 3,

that conferred imidacloprid resistance (Liu *et al.*, 2005). However, this mutation has never, to date, been identified in field resistant strains (Gorman *et al.*, 2008; Puinean, Denholm, *et al.*, 2010). In contrast, there have been multiple studies indicating that heightened cytochrome P450 monooxygenase activity can contribute to neonicotinoid resistance in field-collected populations of *N. lugens* (Liu *et al.*, 2003; Wen *et al.*, 2009). The studies of Liu et al and Wen et al primarily used the metabolic inhibitor PBO in conjunction with imidacloprid to assess the effect on resistant populations. It was seen that there was significant synergism in the resistant strain versus the susceptible (2.93 compared to 1.20). Further evidence implicating P450s in imidacloprid resistance was provided by Puinean et al, using the artificial substrate, 7-ethoxycoumarin, to measure P450 activity in *N. lugens*. Resistant strains displayed ~5-fold higher levels of total activity when compared to the susceptible strain (Puinean et al., 2010).

However, it was not until 2011-2013 that overexpression of individual P450 enzymes, *CYP6ER1* and *CYP6AY1*, were proposed as candidates for imidacloprid resistance (Bass, Carvalho, *et al.*, 2011; Ding *et al.*, 2013). The first study implicated *CYP6ER1* after a screen of 32 tentative unique P450s, that had been identified by two sequencing projects and degenerate PCR. Using qRT-PCR, expression levels of all these P450s was compared between a laboratory susceptible *N. lugens* strain, and moderately and highly resistant populations from China and Thailand, with *CYP6ER1* displaying 40-fold overexpression compared with the susceptible. The resistance of the individual populations was significantly correlated with the level of *CYP6ER1* expression (Bass, Carvalho, *et al.*, 2011). *CYP6AY1*, on the other hand, was seen to be

overexpressed, using qRT-PCR, in a laboratory selected strain. This strain underwent selection with imidacloprid for 40 generations, and 6 out of 14 P450s assessed displayed significant overexpression compared to the susceptible. Of these *CYP6AY1* was the highest at ~18-fold. Further experiments in this study demonstrated that CYP6AY1 could metabolise imidacloprid, and RNAi suggested that *CYP6AY1* could cause resistance to imidacloprid (Ding *et al.*, 2013).

Since 2013, multiple studies have been published on *CYP6ER1* and *CYP6AY1* to attempt to quantify the potential role of each P450 in imidacloprid resistance in *N. lugens*. Upregulation of *CYP6AY1* was suggested to be due to promoter polymorphisms which enhanced the promoter activity of a resistant strain versus a control strain (Pang *et al.*, 2014). A further study directly comparing *CYP6ER1* and *CYP6AY1* concluded that both were important in imidacloprid resistance, with CYP6AY1 the more efficient metaboliser of imidacloprid, but *CYP6ER1* upregulated to a higher level (Bao *et al.*, 2015). One further study published in 2016 performed a functional analysis of *CYP6ER1* (Pang *et al.*, 2016). The main findings of this was that *CYP6ER1* was predominantly expressed in the fat body and midgut, and transgenic expression in *D. melanogaster* causes significant resistance to imidacloprid (Pang *et al.*, 2016).

A novel mechanism of imidacloprid resistance was also proposed in 2015, with down regulation of a target site linked to imidacloprid resistance (Y. Zhang *et al.*, 2015). There were no mutations linked with resistance, but a reduction in Nlα8 nAChR subunit protein levels whilst under imidacloprid selection correlated with increased resistance to imidacloprid. However, as before the resistance described in

the lab-selected strain may not faithfully reflect the primary mechanism of resistance in the field which appears to be P450-mediated.

Chapter V implicated the role of a target-site mutation (A301S) in the RDL GABA receptor with ethiprole resistance, but it is thought that this is not the only mechanism underlying ethiprole resistance in our *N. lugens* populations. Furthermore, two cytochrome P450s (*CYP4DE1* and *CYP6CW3v2*) have been linked with ethiprole resistance in the small brown planthopper (Elzaki, Zhang and Han, 2015).

This chapter discusses the potential roles of *CYP6ER1* and *CYP6AY1* in imidacloprid resistance. Multiple lines of evidences are provided for *CYP6ER1*'s ability to provide resistance to imidacloprid in the *N. lugens* populations that we studied. Furthermore, cross-resistance to the phenylpyrazoles is discussed and potential metabolic mechanisms in ethiprole resistance are evaluated. The cytochrome P450, *CYP4DE1*, is also assessed for a potential role in phenylpyrazole and neonicotinoid resistance.

6.2 Material and Methods

6.2.1 Transgenic expression of cytochrome P450 monooxygenases in *Drosophila melanogaster*

All *CYP6ER1* variants, mutant constructs and *CYP6AY1* were ordered through GeneArt Gene Synthesis (Thermo Fisher, Waltham, MA, USA). *CYP4DE1* was PCR amplified using primers Nl_DE1_BglII_F and Nl_DE1_Xba1_R (Appendix A, Table A6). *CYP6ER1vL*, *CYP6ER1vF* and *CYP6ER1vA* were injected into *D. melanogaster* by the fly facility at the University of Cambridge. All other constructs were ligated into a

modified pattB vector. Microinjections were performed as described previously (Chapter II, Section 2.19). The *Phi*C31 system was utilised to transform the clones into the germ line of a *D. melanogaster* strain containing the attP docking site [y w M(eGFP, vas-int, dmRFP) ZH-2A; P(CaryP)attp40] (Bischof et al., 2007), at a precise chromosomal location on 2L (25C6). Transgenic lines were obtained and balanced (#6126, Df(2L) PMA/SM6a). PCR sequencing confirmed integration of transgenes. The GAL4/UAS system was used to drive expression of the transgene for the bioassays.

6.2.2 High fidelity PCR

When high fidelity PCR products were essential a KAPA HiFi PCR kit (KAPA Biosystems, Boston, MA, USA) was used. Annealing temperatures for HiFi PCR using KAPA are recommended to be between 60-72^{o}C, so primers had to be designed with a higher T_m than for standard PCR (Chapter II, section 2.9). A typical HiFi PCR reaction (25 µL) contained 5 µL 5X KAPA HIFI buffer, 0.75 µL KAPA dNTP mix, 0.75 µL forward primer (10 µM), 0.75 µL reverse primer (10 µM), 2 µL of template cDNA/gDNA and made up to 25 µL with sterile distilled water. Cycling conditions were 95^{o}C for 3 min (initial denaturation) followed by 25-35 cycles of 98^{o}C for 20 s (denaturation), 60-72^{o}C for 20 s (annealing), 72^{o}C for 1 min/kb (extension) and a final extension step of 72^{o}C for 1 min/kb.

6.2.3 Wiggle index

The full protocol for the Wiggle Index (WI) is described in the literature (Denecke *et al.*, 2015) and a condensed version is presented here. The bioassay is broken into several parts.

6.2.3.1 Gathering third instar larvae and insecticide dilution

60 2-5 day old virgin females of line HR-GAL4 (Daborn *et al.*, 2012) were crossed to 20 males containing a P450 transgene under the control of a UAS promoter. The HR-GAL4 strain drives expression in the midgut, Malpighian tubules and fat body when crossed to a strain containing a UAS promoter. Flies were left in the vial of maize meal medium for a period of 24 h at 25^{o}C before being transferred to a fresh vial. Once cleared of adult flies a vial was left for a further 68 h to allow development of a population of 3rd instar larvae. Larvae were harvested from the vial by addition of 30 mL of 20% w/v sucrose, and gentle agitation of the top food layer to free the larvae. These then float to the top of the solution which is then filtered through a fine mesh to recover the larvae, which are then dried and placed on a grape agar plate (Flystuff, San Diego, CA, USA). Here larvae (~5mm in length) were picked and placed into a cell culture 24 well plate (25 larvae per well). The wells contained 200 µL 5% w/v sucrose (Chem Supply, Australia). Larvae were dosed with 50 µL of 5X insecticide stock solution per well, mixed, and then 50 µL of the solution in the well removed. Imidacloprid (200 g L^{-1} Confidor®, Bayer CropScience) was diluted to 120 mg L^{-1} in distilled water to generate the 5X stock solution.

6.2.3.2 Filming

Videos (10 s in duration) were taken at 15, 30, 60 and 120 min after the addition of imidacloprid and these were then compared to videos taken before insecticide addition. Videos captured 4 wells at a time, which were then all processed separately in the downstream analysis. Recording was done by placing the plate on

an LED light box (Huion, Shenzhen, China) and filming with a Panasonic 3CCD Ultra-Compact™ Digital Palmcorder® (Panasonic, Kadoma, Japan).

6.2.3.3 Video processing and analysis

Videos were converted into jpeg images using Video Jpg Converter (DVDVideoSoft), with 250 frames produced per 10 s video. These were then processed on a server using the Fiji distribution of ImageJ (Schindelin *et al.*, 2012) to run the WI script. A basic summary of this script is that it measures movement per well, by assigning values to each pixel's light intensity over the different time points. The precise code and mathematics of this script can be found in (Denecke *et al.*, 2015). This script produced WI values which could then be used to formulate relative movement ratios (RMR). This was done by dividing a WI value at a certain time point by the WI value given for the larvae before the addition on imidacloprid.

6.3 Results

6.3.1 General qRT-PCR of *CYP6ER1* vs *CYP6AY1*

As previously mentioned, there have been two cytochrome P450s implicated in imidacloprid resistant *N. lugens* laboratory and field populations. To assess the potential role of both cytochrome P450s, *CYP6ER1* and *CYP6AY1*, in resistance in the field, expression levels of these two genes were analysed in a range of *N. lugens* populations (primers, Appendix A, Table A5). 12 field populations were tested, sourced from a range of countries in Asia, collected between 2009 and 2012 (Chapter II, Table 2.1). These 12 strains were selected due to their obvious resistance to imidacloprid, highlighted by their reduced mortality in discriminating dose bioassays (Garrood *et al.*, 2016). *CYP6ER1* was consistently significantly overexpressed

compared to the laboratory susceptible strain (Fig. 6.1). All field populations tested demonstrated this overexpression of *CYP6ER1*, ranging from ten- to 90-fold. This was in direct contrast with the expression levels of *CYP6AY1*, which was downregulated in ten of the populations when compared with the laboratory susceptible strain. Only one population showed significant overexpression of *CYP6AY1* - NL59 (3.5-fold). However, the same population demonstrated 71-fold overexpression of *CYP6ER1*. To further investigate the expression of these two genes, and judge whether selection with imidacloprid could increase expression of *CYP6ER1* or *CYP6AY1*, two strains were put under selection pressure as described previously (Chapter III, Section 3.2.3). When the expression level of *CYP6ER1* in NL9 (unselected) and NL9-imi (selected) were compared there had been a significant increase in expression from ~11-fold to 33-fold (compared against the reference susceptible strain). In contrast the expression level of *CYP6AY1* was still downregulated compared to the lab susceptible, even if it had increased from 0.24 in NL9 to 0.29 in Nl9-imi. A similar result was observed for Nl39 (unselected) and NL39-imi (selected), with an increase in *CYP6ER1* expression from 43- to 103-fold. Again, *CYP6AY1* was not significantly overexpressed, only increasing from 0.28 in NL39 to 0.91 in NL39-imi, which was still downregulated compared to the lab susceptible. The 95% confidence limits were significantly reduced in the selected populations when compared with their unselected populations for *CYP6ER1* expression levels.

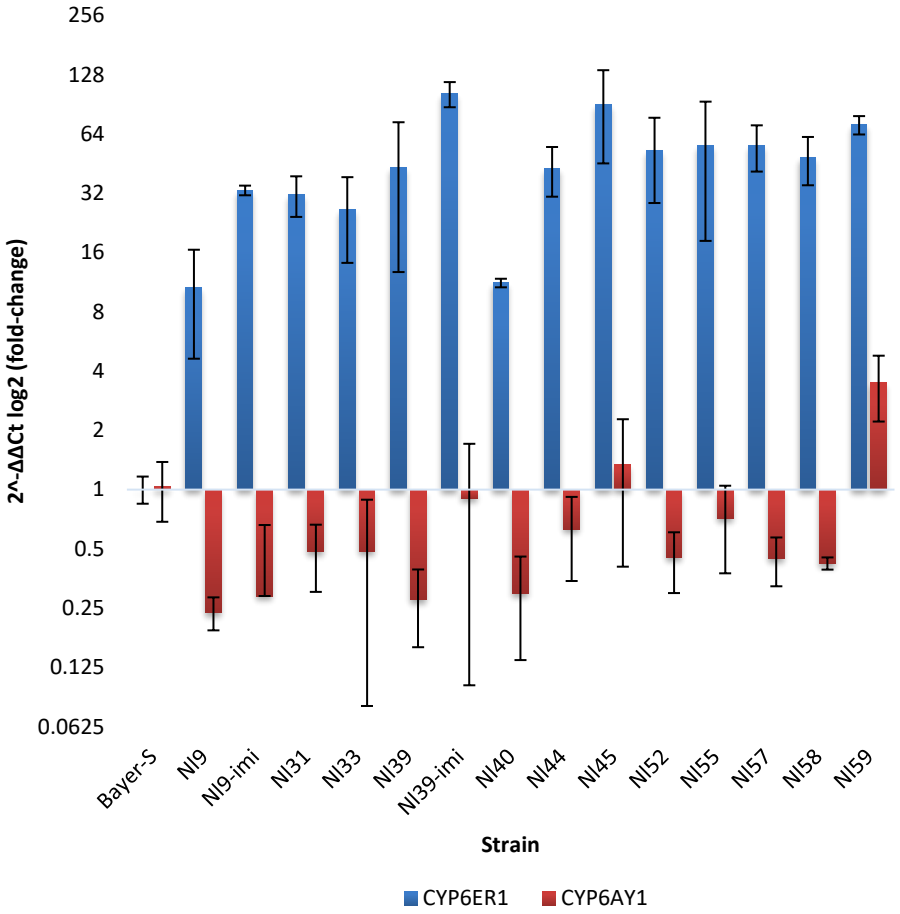


Fig. 6.1 Fold change in expression of *CYP6ER1* and *CYP6AY1* in 14 resistant *N. lugens* strains compared with the susceptible reference Bayer-S as determined by qRT-PCR. Error bars display 95% confidence intervals.

Since the values for *CYP6AY1* differed remarkably from those previously reported (Ding *et al.*, 2013), the primer pair used in that study was also tested. The populations in Table 6.1 were retested by qRT-PCR to assess expression of *CYP6AY1*. The result of this concurred with the initial findings (Fig 6.1), with all the populations being downregulated when compared with the lab susceptible, including the two imidacloprid selected strains.

Table 6.1 Fold change in expression of *CYP6AY1* in five imidacloprid-resistant *N. lugens* strains compared with the susceptible reference Bayer-S as determined by qRT-PCR.

Strain	Fold Change (2^-ΔΔCt)	95% confidence level
Bayer-S	1.055	0.451
Nl9	0.296	0.096
Nl9-imi	0.203	0.284
Nl39	0.251	0.062
Nl39-imi	0.504	0.343
Nl59	0.783	0.345

6.3.2 Sequencing of *CYP6ER1*

Previous sequencing of *CYP6ER1* had revealed amino acid substitutions between the imidacloprid resistant strains and the susceptible strain (Bass, Carvalho, *et al.*, 2011). Resequencing of this gene (primers, Appendix A, Table A6) revealed new variants that had not been seen in the previous study performed by Bass et al. The sequencing was performed on pools of cDNA from a broad range of *N. lugens* populations. This included populations that had been selected on ethiprole in the laboratory up to 100 mg L^{-1}. The result of this was curation of eight unique coding sequences seen from the sequencing of *CYP6ER1* (Fig. 6.2). *CYP6ER1vL* is the sequence derived from the laboratory susceptible strain, and is displayed here as the reference sequence. *CYP6ER1vF* was originally seen in the Nl9 population, the imidacloprid resistant population previously that displayed overexpression of *CYP6ER1* (Bass, Carvalho, *et al.*, 2011). The remaining six variants; *CYP6ER1vA*, *CYP6ER1vB*, *CYP6ER1vC*, *CYP6ER1vD1*, *CYP6ER1vD2* and *CYP6ER1vE* were new variants that had not previously been seen. Two of these variants showed a particularly profound alteration, both *CYP6ER1vA* and *CYP6ER1vB*, differed from the other variants in that they have a 3bp deletion resulting in the deletion of a single amino acid as well as multiple other substitutions. For *CYP6ER1vA* this deletion

occurs at AA 375 (alanine), and is immediately followed by an additional amino acid substitution, A376G. This deletion and substitution is in the predicted 5th substrate recognition site (SRS-5). *CYP6ER1vB* also contains a deletion in SRS-5, however is at 377 (proline) and has no substitution at 376.

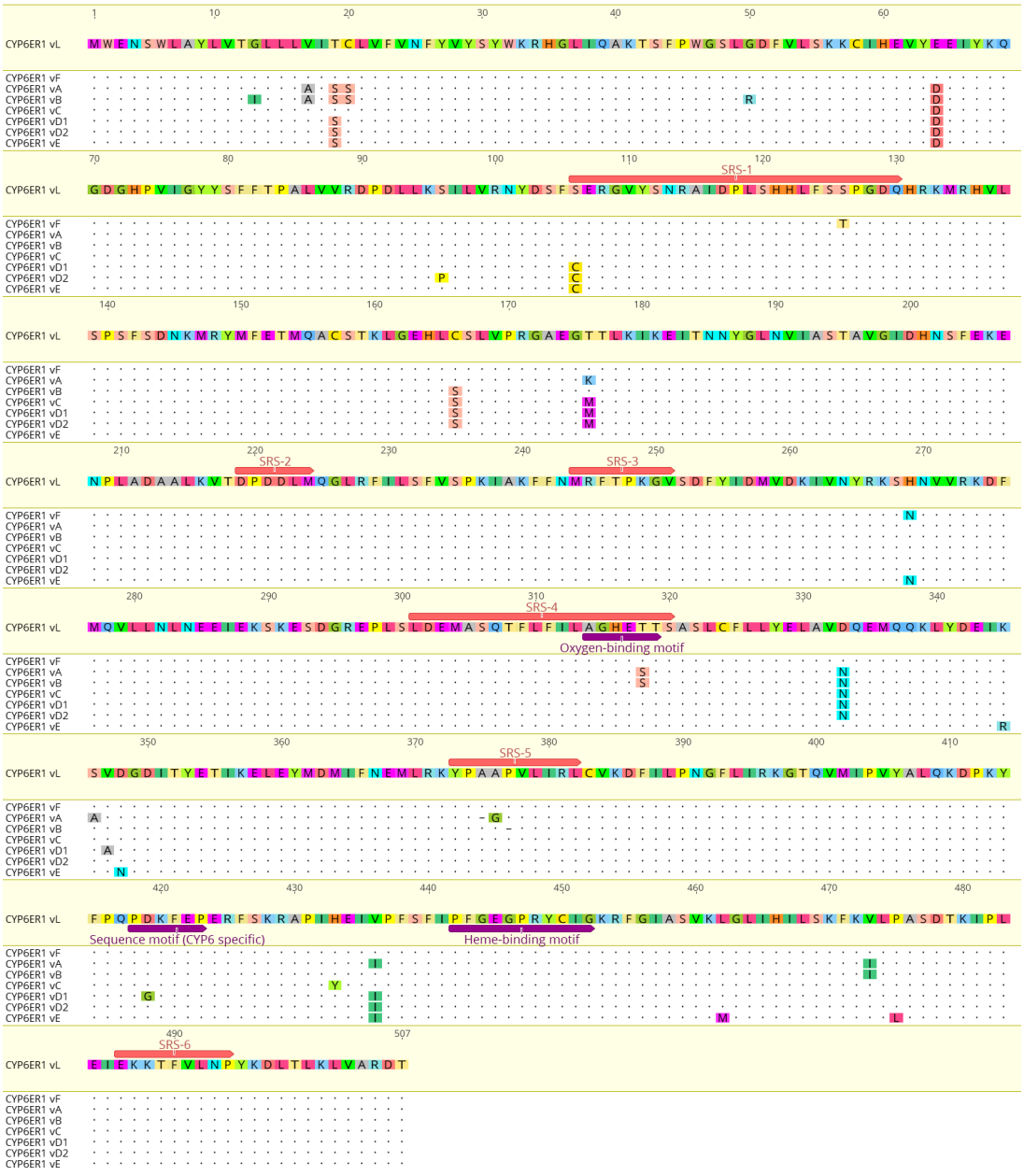


Fig. 6.2 Amino acid alignment of the *CYP6ER1* gene sequence from the laboratory susceptible and imidacloprid resistant *N. lugens* strains. Dots represent identical residues. Deletions are represented by dashes and substitutions are highlighted. Conserved domains to cytochrome P450 monooxygenases are indicated (oxygen binding motif and the heme-binding motif). The proposed substrate recognition sites (SRSs) are also marked.

6.3.3 Variant specific qRT-PCR of *CYP6ER1*

Since the qRT-PCR analysis displayed in Fig. 6.1 was using a generic primer pair for *CYP6ER1*, it only gave information on overexpression of the predominant *CYP6ER1* variant expressed in each strain of *N. lugens* tested. The sequencing of *CYP6ER1* demonstrated that there were far more variants than previously thought in the *N. lugens* populations (Fig. 6.2). However, expression of each individual variant had not been analysed at this point. To address this primer pairs were designed that were specific to a *CYP6ER1* variant (Appendix A, Table A7 and Fig. A1), and qRT-PCR was performed. This was performed for ten *N. lugens* populations and the laboratory susceptible as a reference (Fig. 6.3). The qRT-PCR results are displayed as 1/ΔCt. It was not possible to calculate 2^-ΔΔCt values, because some of the variants were not expressed in the reference strain (Bayer-S). The 1/ΔCt values allow a comparison of which *CYP6ER1* variants are most expressed in the resistant strains. The first observation from the qRT-PCR is that despite there being eight sequenced variants of *CYP6ER1*, only two of these are commonly highly expressed in the *N. lugens* field populations sampled. These two variants are *CYP6ER1vA* and *CYP6ER1vB*, the two variants with the AA deletion in SRS-5. Nl9 and Nl9-imi showed low levels of expression of *CYP6ER1vF*, but significantly higher expression of *CYP6ER1vA* in comparison. Similarly, Nl39 and Nl39-imi both showed greater expression of *CYP6ER1vA* than any other *CYP6ER1* variant. As with the previous qRT-PCR (Section 6.3.1), there were higher levels of expression of *CYP6ER1* (*CYP6ER1vA* in this case) in the imidacloprid selected populations compared to their unselected counterparts. Interestingly this phenomenon was also seen when comparing ethiprole selected populations with their unselected populations (Nl33 vs Nl33-eth/Nl39 vs Nl39-eth).

Nl55 and Nl55-eth differed from the other populations in that they over-expressed *CYP6ER1vB*, rather than *CYP6ER1vA*.

Each resistant *N. lugens* population only significantly expresses one of the main *CYP6ER1* variants, since there are no populations that significantly express both *CYP6ER1vA* and *CYP6ER1vB*. Furthermore, the expression of *CYP6ER1vA or CYP6ER1vB* can be linked to geographical location. *CYP6ER1vB* was seen in Nl55 (India) and analysis of another Indian population (Nl59) revealed it also expressed *CYP6ER1vB*. In contrast *CYP6ER1vA* was seen in populations from Thailand (Nl9) and Vietnam (Nl33 and Nl39).

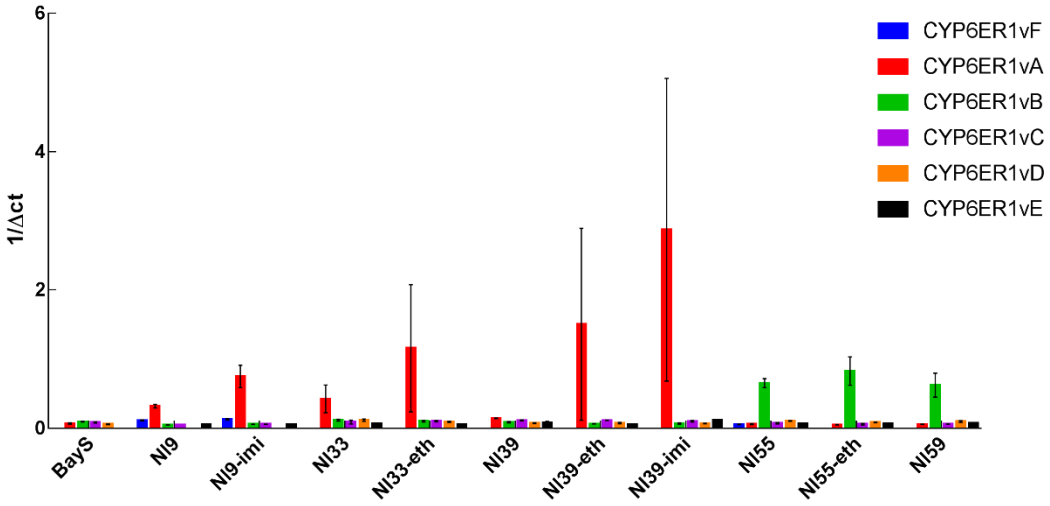


Fig. 6.3 1/ΔCt values of *CYP6ER1* variants in ten imidacloprid and ethiprole resistant strains and the laboratory susceptible as determined by qRT-PCR.

6.3.4 RNA-seq read mapping to *CYP6ER1* variants

To further explore which *CYP6ER1* variants were seen in which *N. lugens* populations the RNA-seq reads described in Chapter IV were mapped back to variant-specific *CYP6ER1* fragments of sequence. The reads that aligned to *CYP6ER1vF* were so

limited (Table 6.2) that it is probable that this variant is not being expressed in any of these populations, supporting results seen in the qRT-PCR (Section 6.3.3). The findings for *CYP6ER1vA* and *CYP6ER1vB* also agree with the trend seen in the qRT-PCR. Nl33 and Nl33-eth aligned significantly only to the *CYP6ER1vA* fragment, with some reads in the Nl55 and Nl55-eth populations aligning to this fragment also. However, the number of reads aligning to *CYP6ER1vB* for Nl55 and Nl55-eth was far higher than for the *CYP6ER1vA*.

Table 6.2 RNA-seq reads aligned to *CYP6ER1* variants.

	Reads aligned				
Variant	Bayer-S	Nl33	Nl33-eth	Nl55	Nl55-eth
CYP6ER1vL	644	0	0	3	0
CYP6ER1vF	53	3	1	11	2
CYP6ER1vA	15	5016	15594	856	435
CYP6ER1vB	11	24	18	16659	12045

6.3.5 *D. melanogaster* bioassays of *CYP6ER1* variants and *CYP6AY1* strains

Functional expression of *CYP6ER1* variants was performed to analyse whether there was a qualitative effect as well as the quantitative effect demonstrated in the qRT-PCR studies. The *CYP6ER1* variants tested were *CYP6ER1vL*, *CYP6ER1vF*, *CYP6ER1vA*, *CYP6ER1vB* and *CYP6ER1vC*. The remaining variants (*CYP6ER1vD1*, *CYP6ER1vD2* and *CYP6ER1vE*) were rejected for functional analysis since there was no noticeable expression of any of these three variants in the qRT-PCR analysis compared to the susceptible strain. *CYP6AY1* was also included in the functional analysis, to assess whether it could have any impact on insecticide resistance.

Against imidacloprid (Fig 6.4 andTable 6.3), there was a significant difference in resistance across the variants tested. *CYP6ER1vA* and *CYP6ER1vB* had similar LC_{50}s

of 608 and 579 respectively, which equated to a RR of ~4.5 against the control strain. *CYP6ER1vF* also showed partial resistance, with a RR of 2.55 at LC_{50}. However, *CYP6AY1* was only marginally more resistant than the control strain (RR 1.7), as was *CYP6ER1vC* (RR 1.2). The variant from the susceptible (Bayer-S), *CYP6ER1vL* was much lower than the control (RR 0.4).

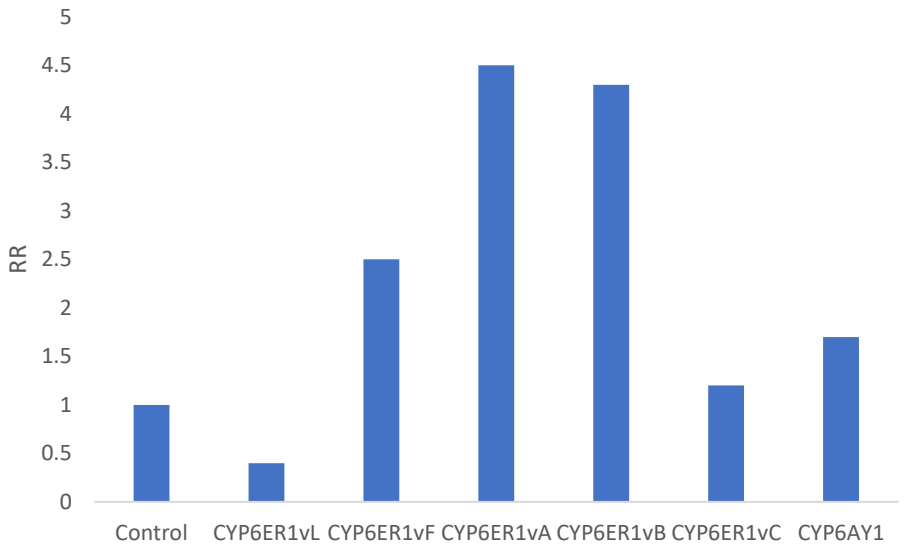


Fig 6.4 Resistance ratios for *D. melanogaster* strains against imidacloprid

Table 6.3 Log-concentration probit mortality data for imidacloprid against *D. melanogaster* strains containing *CYP6ER1* variants or *CYP6AY1.*

Compound	Strain	LC_{50} [mg/L^{-1}]	95% CL	Slope (± SD)	Resistance Ratio To Control	Resistance Ratio To CYP6ER1vL
Imidacloprid	Control	135	36-330	0.888 ± 0.167	1.0	2.5
	CYP6ER1vL	53.1	31.4-82.6	1.864 ± 0.328	0.4	1.0
	CYP6ER1vF	344	275.6-431	2.164 ± 0.187	2.5	6.5
	CYP6ER1vA	608	333-1111	1.721 ± 0.306	4.5	11.5
	CYP6ER1vB	579	439-761	2.416 ± 0.823	4.3	10.9
	CYP6ER1vC	168.4	82.6-339	2.046 ± 0.474	1.2	3.2
	CYP6AY1	223.6	135.4-366	1.846 ± 0.297	1.7	4.2

Thiacloprid testing revealed only one strain showing a significantly high RR at LC_{50}, which was *CYP6ER1vB* at 3.82 (Fig. 6.5 and Table 6.4). *CYP6ER1vA* was only slightly

more resistant than the control (RR 1.58). All other strains displayed no resistance compared to the control at all.

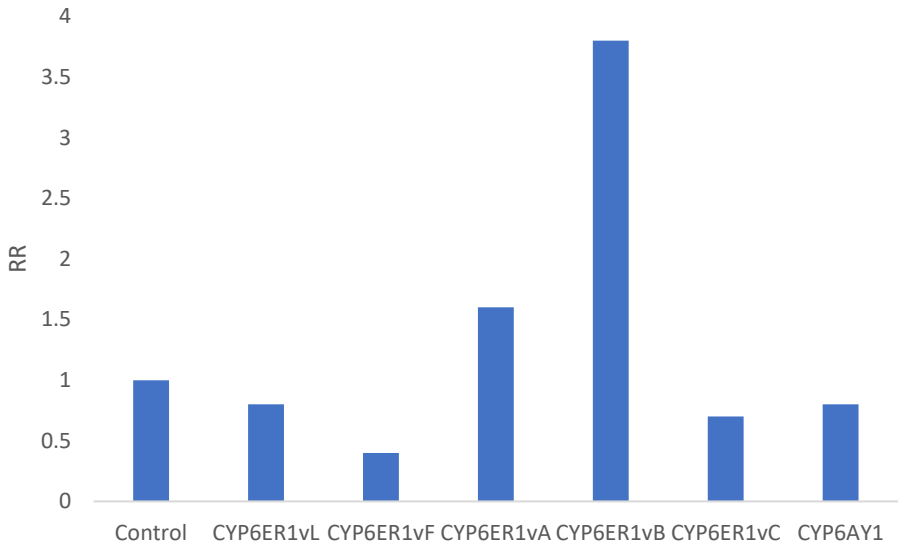


Fig. 6.5 Resistance ratios for *D. melanogaster* strains against thiacloprid

Table 6.4 Log-concentration probit mortality data for thiacloprid against *D. melanogaster* containing *CYP6ER1* variants or *CYP6AY1.*

Compound	Strain	LC_{50} [mg/L^{-1}]	95% CL	Slope (± SD)	Resistance Ratio To Control	Resistance Ratio To CYP6ER1vL
Thiacloprid	Control	553	358-824	2.153 ± 0.366	1.0	1.2
	CYP6ER1vL	442.6	nc	6.4 ± 16.2	0.8	1.0
	CYP6ER1vF	216	110-398	1.154 ± 0.174	0.4	0.5
	CYP6ER1vA	871	677-1122	2.069 ± 0.194	1.6	2.0
	CYP6ER1vB	2115	1420-3174	2.056 ± 0.304	3.8	4.8
	CYP6ER1vC	360	116-987	1.303 ± 0.319	0.7	0.8
	CYP6AY1	438	287-664	1.826 ± 0.246	0.8	1.0

The response to nitenpyram (Fig. 6.6 and Table 6.5) was different from that of imidacloprid and thiacloprid, with strain *CYP6ER1vL* showing the highest LC_{50} (446, RR 2.6). Strain *CYP6ER1vA* had an LC_{50} of 457, however the lack of 95% confidence limits undermines the reliability of the LC_{50}. When there is large variation between the biological replicates it is not always possible to formulate 95% confidence limits

in probit analysis. Also, if there is a large change in mortality between two concentrations (taking the response far from the linear relationship expected) then 95% confidence limits will be incalculable. Of the other strains *CYP6ER1vB*, *CYP6ER1vC* and *CYP6AY1* all had similar LC_{50}, but *CYP6ER1vF* had no significant resistance.

Strains *CYP6ER1vA* and *CYP6ER1vB* showed resistance to clothianidin, RRs of 5.1 and 4.3 at LC_{50} respectively. *CYP6AY1* and *CYP6ER1vL* also demonstrated some resistance (RR 3 and 2.8 at LC_{50}), but *CYP6ER1vF* showed no resistance compared to the control. Dinotefuran testing demonstrated no strain displaying high resistance. *CYP6ER1vL* had the highest RR of 2 at LC_{50}. Thiamethoxam saw a similar trend to clothianidin, with *CYP6ER1vA* having the highest RR at 5.9 at LC_{50}, followed by *CYP6ER1vB* (RR 3.4) and *CYP6ER1vL* (RR 3.3). Acetamiprid was unique, in that no strain displayed any resistance, all having LC_{50}s below that of the control strain.

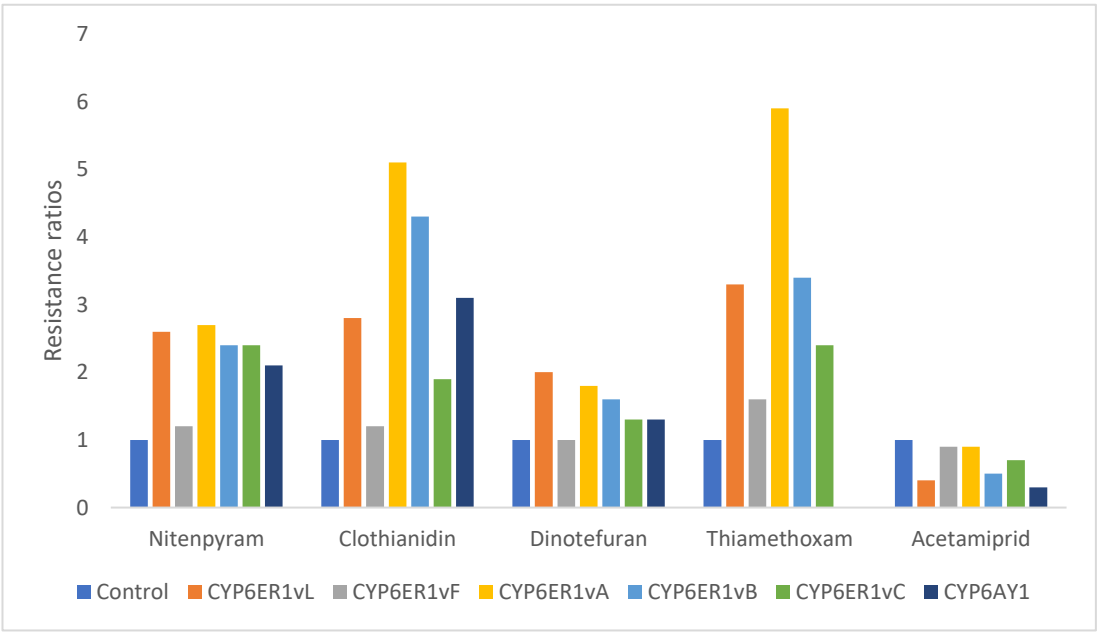


Fig. 6.6 Resistance ratios for *D. melanogaster* strains against the other neonicotinoids

Table 6.5 Log-concentration probit mortality data for other neonicotinoids against *D. melanogaster* containing *CYP6ER1* variants or *CYP6AY1*.

Compound	Strain	LC_{50} [mg/L^{-1}]	95% CL	Slope (± SD)	Resistance Ratio To Control	Resistance Ratio To CYP6ER1vL
Nitenpyram	Control	171	142.2-206.2	5.322 ± 0.923	1.0	0.4
	CYP6ER1vL	446.1	370.4-537.5	5.389 ± 0.929	2.6	1.0
	CYP6ER1vF	205.3	120-337	2.962 ± 0.738	1.2	0.5
	CYP6ER1vA	457.8	nc	0	2.7	1.0
	CYP6ER1vB	417.3	287.9-602	3.555 ± 0.756	2.4	0.9
	CYP6ER1vC	405.5	280.8-586	3.56 ± 0.754	2.4	0.9
	CYP6AY1	366.1	293-457	3.925 ± 0.588	2.1	0.8
Clothianidin	Control	5.07	nc	8.2 ± 13.8	1.0	0.4
	CYP6ER1vL	14.05	nc	8.4 ± 16.2	2.8	1.0
	CYP6ER1vF	6.02	5.3-6.9	3.516 ± 0.324	1.2	0.4
	CYP6ER1vA	26.02	nc	7.1 ± 11.6	5.1	1.9
	CYP6ER1vB	21.66	nc	7.3 ± 10.5	4.3	1.5
	CYP6ER1vC	9.54	5.9-15.2	3.44 ± 0.801	1.9	0.7
	CYP6AY1	15.48	10-23.7	2.254 ± 0.39	3.1	1.1
Dinotefuran	Control	22.32	nc	7.1 ± 13.8	1.0	0.5
	CYP6ER1vL	44.72	37.4-53.5	5.247 ± 0.527	2.0	1.0
	CYP6ER1vF	21.66	nc	7.3 ± 10.5	1.0	0.5
	CYP6ER1vA	41.27	35.2-48.8	4.771 ± 0.446	1.8	0.9
	CYP6ER1vB	35.72	31.9-40.1	3.38 ± 0.22	1.6	0.8
	CYP6ER1vC	28.77	nc	8.1 ± 11.3	1.3	0.6
	CYP6AY1	29.71	nc	8.7 ± 14.2	1.3	0.7
Thiamethoxam	Control	9.69	8.2-11.4	4.771 ± 0.445	1.0	0.3
	CYP6ER1vL	31.63	26.3-38.4	3.689 ± 0.435	3.3	1.0
	CYP6ER1vF	15.42	nc	7.4 ± 12.2	1.6	0.5
	CYP6ER1vA	56.7	32.8-97.9	2.181 ± 0.431	5.9	1.8
	CYP6ER1vB	33.2	28.5-39.1	3.839 ± 0.379	3.4	1.0
	CYP6ER1vC	23.56	nc	7.4 ± 11.7	2.4	0.7
	CYP6AY1	16.4	12.9-20.9	2.436 ± 0.26	1.7	0.5
Acetamiprid	Control	131	nc	7.2 ± 11	1.0	2.7
	CYP6ER1vL	47.9	21.4-129	2.368 ± 0.686	0.4	1.0
	CYP6ER1vF	121.6	94-149.9	2.99 ± 0.374	0.9	2.5
	CYP6ER1vA	122	nc	7.2 ± 12.6	0.9	2.5
	CYP6ER1vB	63.92	nc	9.4 ± 14.1	0.5	1.3
	CYP6ER1vC	93.5	67.5-129.3	3.038 ± 0.525	0.7	2.0
	CYP6AY1	41.27	33.4-51.7	4.771 ± 0.588	0.3	0.9

Since it was seen that selection with ethiprole could cause *CYP6ER1* expression to increase compared to an unselected population, it was decided to test the *CYP6ER1* variants and *CYP6AY1* against the fiproles. In the ethiprole bioassay (Fig. 6.7), *CYP6ER1vA* showed high resistance (RR 6.9), whereas all the other *CYP6ER1* variants had LC_{50}s below that of the control strain (Table 6.6). *CYP6AY1* had a RR of 1.8 at LC_{50}, however this was still ~4-fold lower than that seen by *CYP6ER1vA*.

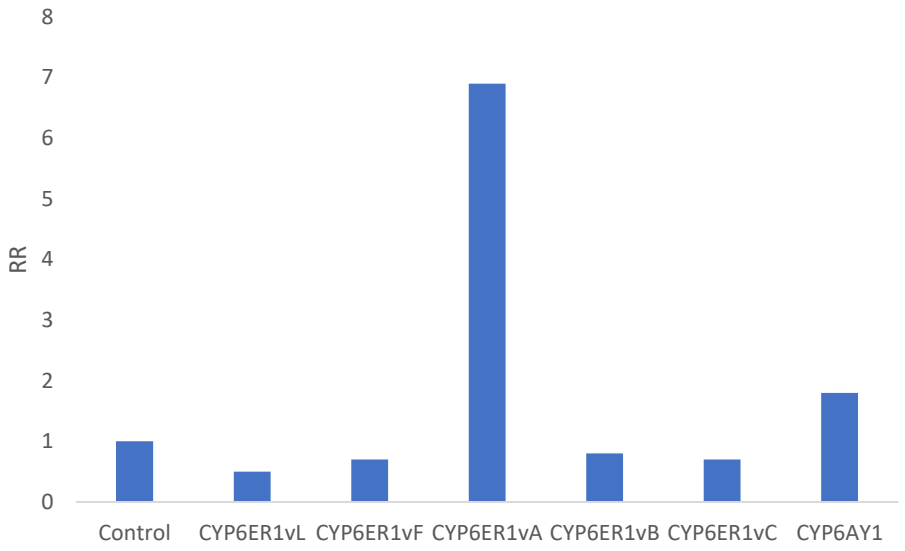


Fig. 6.7 Resistance ratios for *D. melanogaster* strains against ethiprole

Table 6.6 Log-concentration probit mortality data for ethiprole against *D. melanogaster* containing *CYP6ER1* variants or *CYP6AY1*.

Compound	Strain	LC_{50} [mg/L^{-1}]	95% CL	Slope (± SD)	Resistance Ratio To Control	Resistance Ratio To CYP6ER1vL
Ethiprole	Control	53.1	24.3-110	1.325 ± 0.245	1.0	2.2
	CYP6ER1vL	23.9	11.3-42.1	1.452 ± 0.244	0.5	1.0
	CYP6ER1vF	37.61	33.2-42.7	3.546 ± 0.257	0.7	1.6
	CYP6ER1vA	369	11.7-1115	1.431 ± 0.542	6.9	15.4
	CYP6ER1vB	44.39	36.7-53.6	3.181 ± 0.32	0.8	1.9
	CYP6ER1vC	37.4	25.8-54	2.007 ± 0.265	0.7	1.6
	CYP6AY1	94.9	68.7-131.1	2.387 ± 0.319	1.8	4.0

In comparison, none of the strains demonstrated any resistance against fipronil (Fig. 6.8 and Table 6.7). It was not possible to calculate an LC_{50} for *CYP6ER1vA*,

since it saw full mortality at all tested doses of fipronil. There was also considerable difference in the lethality between ethiprole and fipronil, fipronil producing a far lower LC_{50} for the controlled compared to ethiprole, 2.695 vs 53.1 respectively.

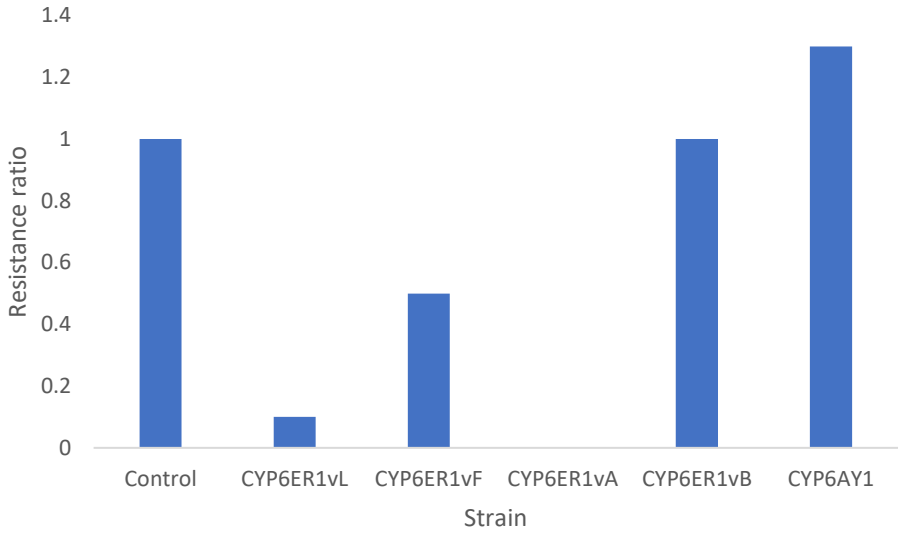


Fig. 6.8 Resistance ratios for *D. melanogaster* strains against fipronil

Table 6.7 Log-concentration probit mortality data for fipronil against *D. melanogaster* containing *CYP6ER1* variants or *CYP6AY1*.

Compound	Strain	LC_{50} [mg/L^{-1}]	95% CL	Slope (± SD)	Resistance Ratio To Control	To CYP6ER1vL
Fipronil	Control	2.695	nc	4.9 ± 13.3	1.0	9.0
	CYP6ER1vL	0.299	0.002-1.07	1.068 ± 0.304	0.1	1.0
	CYP6ER1vF	1.441	nc	4.1 ± 12.9	0.5	4.8
	CYP6ER1vA	Nc	nc	nc	nc	nc
	CYP6ER1vB	2.638	nc	5.3 ± 11.1	1.0	8.8
	CYP6AY1	3.371	nc	5.8 ± 12.4	1.3	11.3

6.3.6 Wiggle index bioassays

The *CYP6ER1* variants and *CYP6AY1* were overexpressed in the midgut, Malpighian tubules and fat body and tested with 24 mg L^{-1} imidacloprid. A line that overexpressed *CYP6ER1vA* showed significantly less response to imidacloprid than compared to a line overexpressing *CYP6ER1vL* (Fig 6.9).

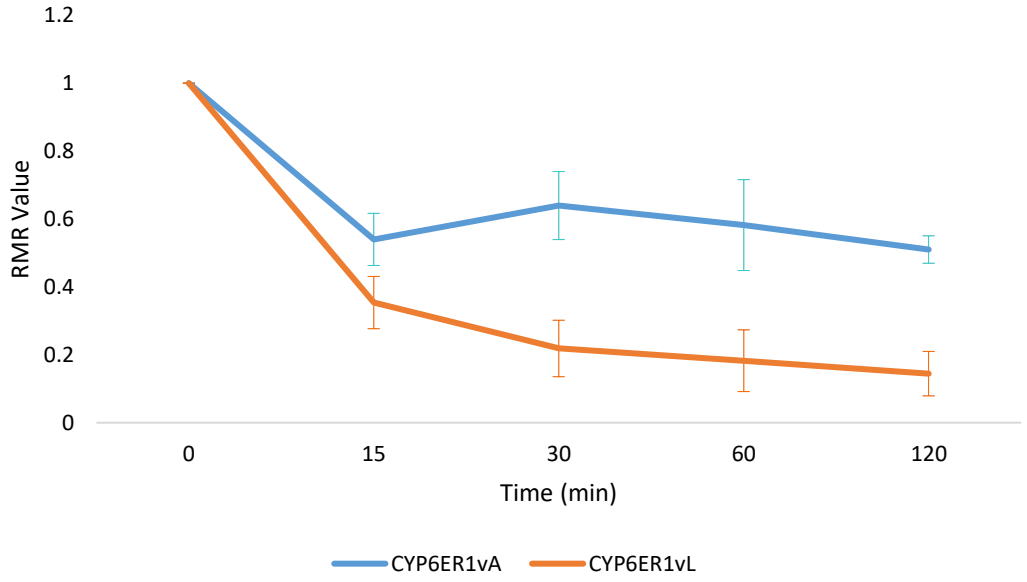


Fig. 6.9 Response curves for *CYP6ER1vA* and *CYP6ER1vL* after treatment with 24 mg L^{-1} imidacloprid. Points represent mean RMR values with 95% confidence intervals.

In comparison a line overexpressing *CYP6AY1* was only significantly different from the *CYP6ER1vL* line at one timepoint (60 min). At all other timepoints it overlapped with the *CYP6ER1vL* line in its response to imidacloprid (Fig. 6.10).

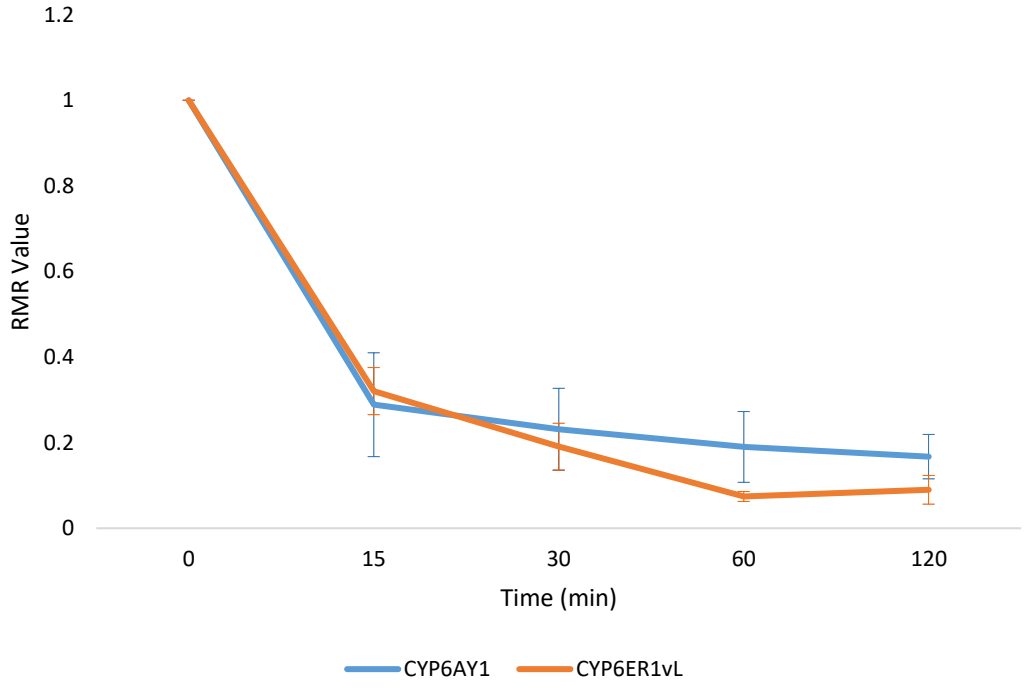


Fig. 6.10 Response curves for *CYP6AY1* and *CYP6ER1* vS after treatment with 24 mg L^{-1} imidacloprid. Points represent mean RMR values with 95% confidence intervals.

In contrast to the previous wiggle indexes (Fig. 6.9 and Fig. 6.10) the final wiggle index bioassay did not give a clear trend. For previously discussed wiggle indexes there was a large drop in RMR between the 0 and 15 min time points. However, this was not seen for the bioassay involving *CYP6ER1vB*, *CYP6ER1vF* and *CYP6ER1vL* (Fig. 6.11). *CYP6ER1vL* took significantly longer to see a reduction in movement after imidacloprid exposure, and its final RMR is approximately three-fold higher than previous bioassays. Likewise, *CYP6ER1vB* and *CYP6ER1vF* appear broadly unaffected by the addition of imidacloprid, with the RMR at 120 min being near to that of 0 min. It would appear something went wrong with this bioassay run and so is difficult to state whether there is a significant difference between *CYP6ER1vB* and *CYP6ER1vF* compared to the susceptible line here.

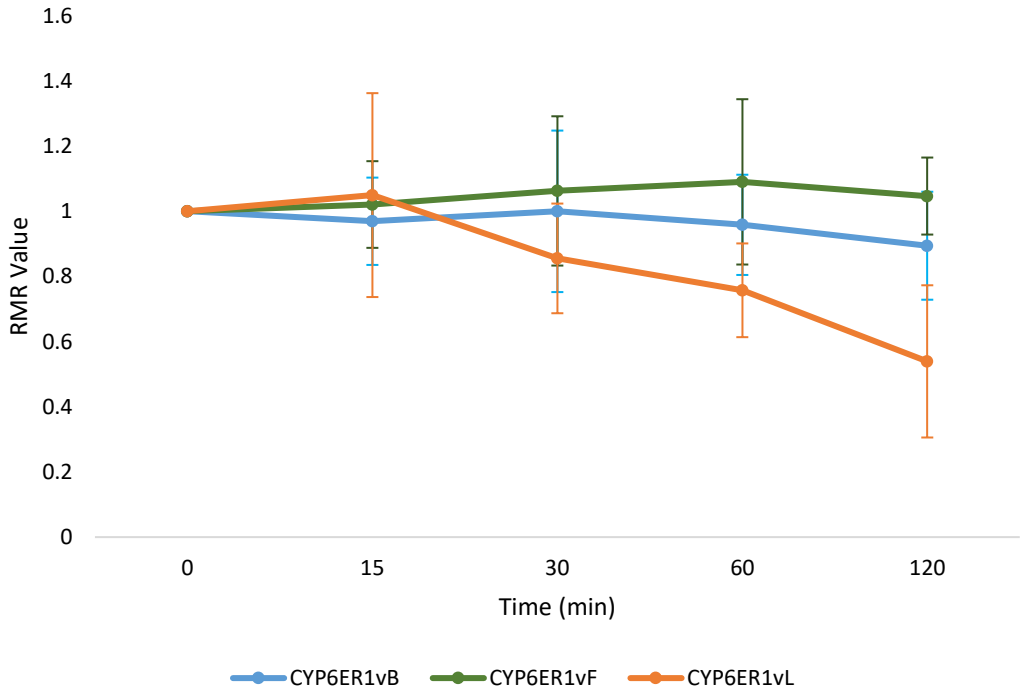


Fig. 6.11 Response curves for *CYP6ER1vB*, *CYP6ER1vF* and *CYP6ER1vL* after treatment with 24 mg L^{-1} imidacloprid. Points represent mean RMR values with 95% confidence intervals.

6.3.7 Locomotor activity level monitoring with imidacloprid and ethiprole

Five strains (*CYP6ER1vL, CYP6ER1vF, CYP6ER1vA, CYP6ER1vB* and *CYP6AY1*) were tested further using locomotor activity monitoring, to assess resistance to imidacloprid and ethiprole. *CYP6ER1vL, CYP6ER1vF* and *CYP6ER1vB* all showed a reduction in activity and no longer followed the profiles of their respective controls when exposed to imdiacloprid (Fig. 6.12). However, *CYP6ER1vA* showed a similar profile to its control displaying that this strain was not adversly affected by imidacloprid, as shown in the wiggle index and fly mortality bioassays. *CYP6AY1* was also able to maintain activity levels similar to the control, but to a lesser extent than seen for *CYP6ER1vA*.

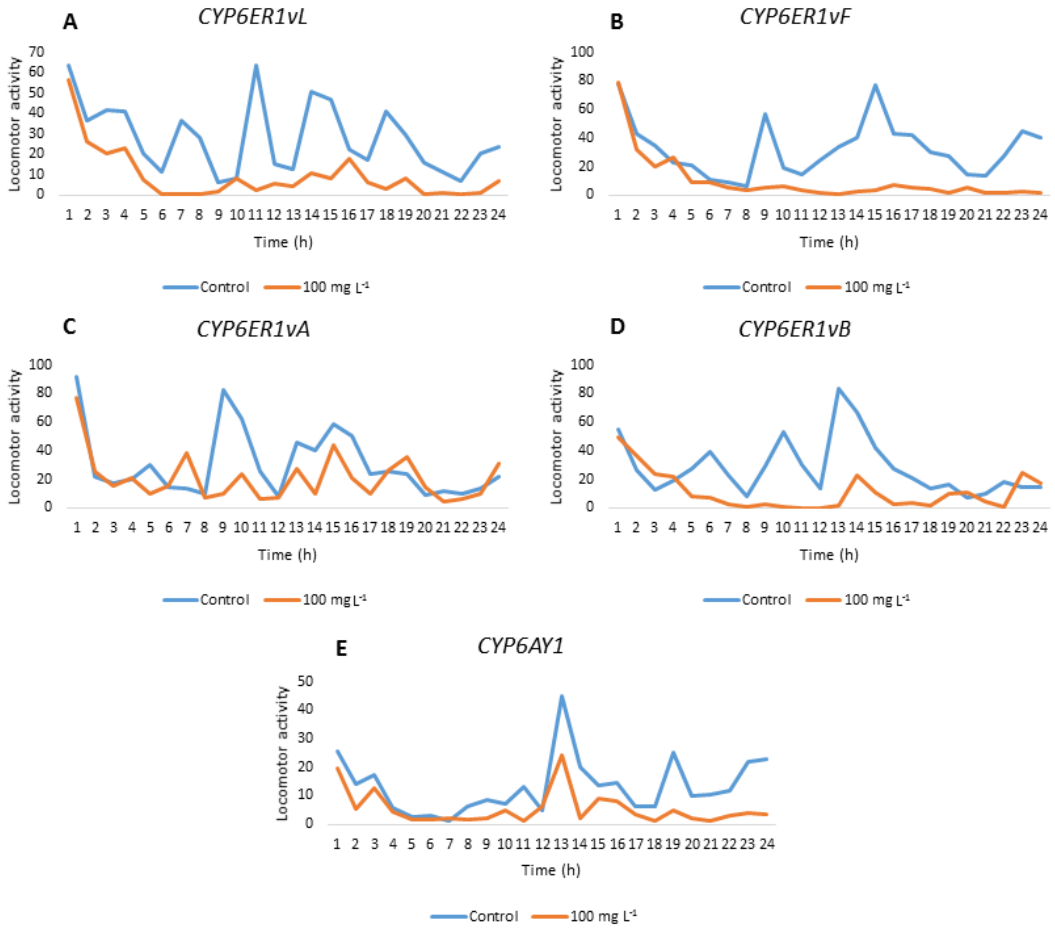


Fig. 6.12 Locomotor activity of *CYP6ER1* variants and *CYP6AY1* when exposed to 100 mg L^{-1} imidacloprid. Time displays the number of hours the flies have been on the imidacloprid treated food. Locomotor activity displayed is the average movement recorded for each of the 5 min sections within a given hour.

When four *CYP6ER1* variants were exposed to ethiprole there was a significant difference in locomotor activity displayed. *CYP6ER1vL* and *CYP6ER1vF* were both disrupted and within 40 hours both strains displayed no movement, whilst their respective controls were still active. *CYP6ER1vA,* the only strain to display resistance to ethiprole in the fly mortality bioassays, again showed an ability to survive ethiprole exposure (Fig. 6.13). This strain broadly tracks the profile of it's control and at no point is there zero movement recorded. Surprisingly *CYP6ER1vB* also showed

resistance to ethiprole, and was able to remain active throughout the 72 h recording period.

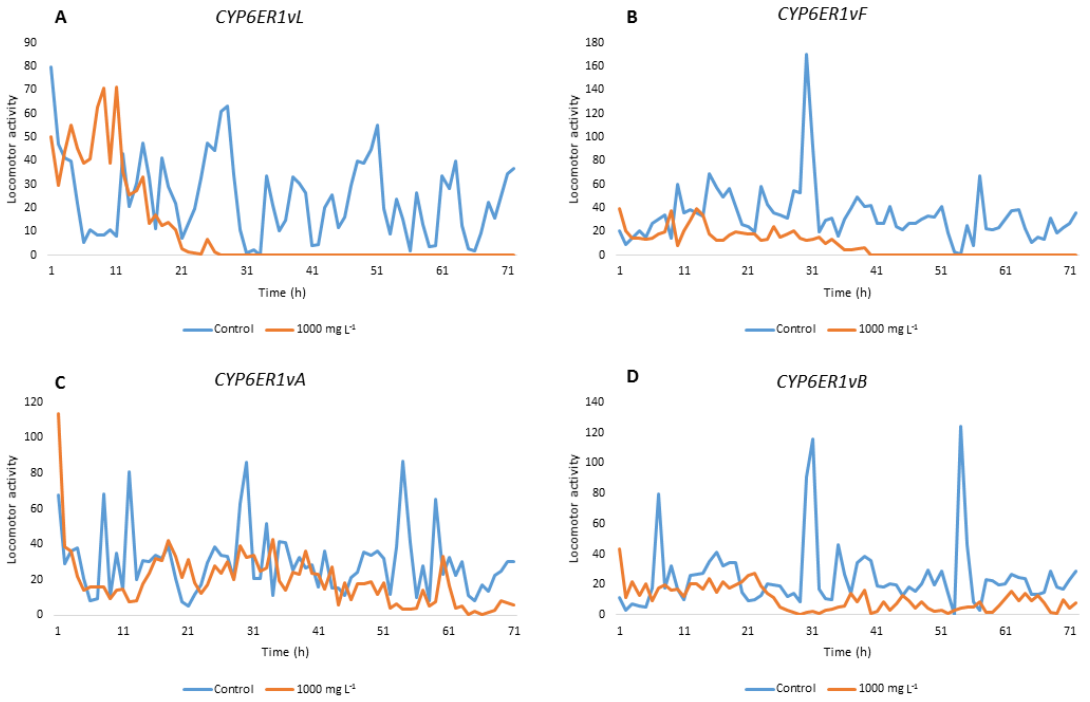


Fig. 6.13 Locomotor activity of *CYP6ER1* variants when exposed to 1000 mg L^{-1} ethiprole. Time displays the number of hours that the flies have been on the ethiprole coated food. Locomotor activity displayed is the average movement recorded for each of the 5 min sections within a given hour.

6.3.8 Mutant *CYP6ER1* constructs and *D. melanogaster* bioassays

The results of the variant specific qRT-PCR and *D. melanogaster* bioassays showed that *CYP6ER1vA* and *CYP6ER1vB* are the key resistance conferring variants. The coding sequence of these genes were analysed by mapping known important conserved P450 motifs and substrate recognition sites in order to predict which AA substitutions/deletions are likely to confer resistance to imidacloprid. This suggested the deletions/substitutions occurring in SRS-5 and a further T to S change in the SRS-4 which was only seen in *CYP6ER1vA* and *CYP6ER1vB* were strong candidates as gain-of-function mutations. To test this, mutant constructs were created that introduced

various combinations of the deletions/substitutions of predicted importance in imidacloprid resistance. The alterations were made to *CYP6ER1vL*, which had shown far lower resistance to imidacloprid in the preceding *D. melanogaster* insecticide bioassays. The five constructs created are shown (Fig. 6.14), in comparison with the *CYP6ER1vL*, *CYP6ER1vA* and *CYP6ER1vB* sequences.

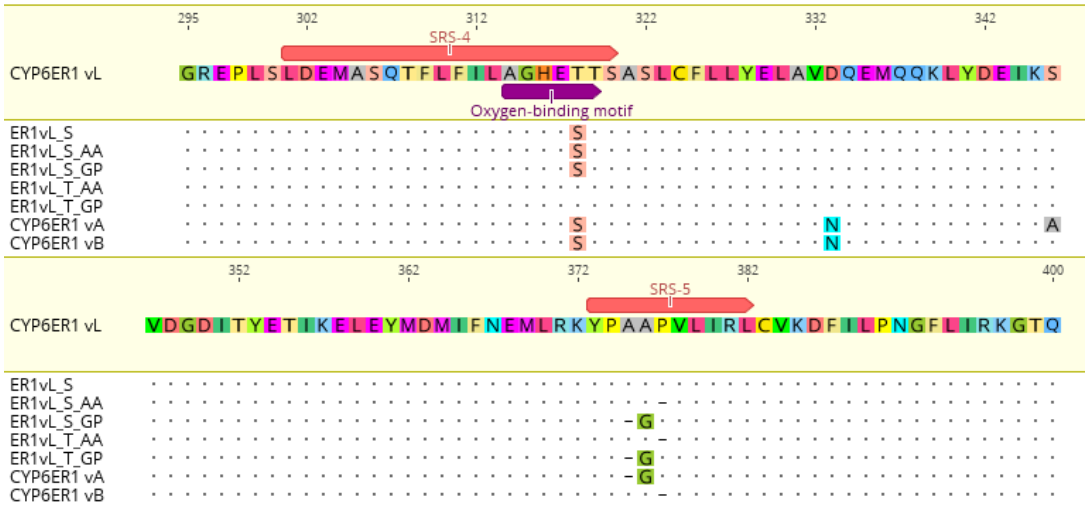


Fig. 6.14 Amino acid alignment highlighting the mutant constructs created with the mutations introduced to *CYP6ER1vL.*

The constructs generated (Fig. 6.14) were injected into *D. melanogaster* and bioassays were performed with seven neonicotinoids and ethiprole, with the help of a postdoctoral scientist and technician. Of the seven neonicotinoids used, the response to imidacloprid was the most striking. Three of the strains produced near identical LC_{50} values and gave RRs of 20-fold against *CYP6ER1vL* strain (Table 6.8). These were ER1vL_S_AA (*CYP6ER1vB*-like), ER1vL_S (serine substitution in SRS-4 only, no other mutations) and ER1vL_T_GP (No serine substitution in SRS-4, *CYP6ER1vA* deletion and substitution in SRS-5). Of the two remaining mutant constructs, ER1vL_T_AA (no serine substitution in SRS-4, CYP6ER1vB deletion in SRS-

5) had a lower RR at LC_{50} of 4.5, whereas ER1vL_S_GP (*CYP6ER1vA*-like) had a very high RR at LC_{50} of 35.

For thiacloprid there was a lower level of resistance, compared to imidacloprid, for all the mutant constructs (Fig. 6.15 and Table 6.9). ER1vL_S_AA and ER1vL_T_AA had LC_{50}s near identical to each other, whereas the most resistant strain against imidacloprid, ER1vl_S_GP, had lower resistance than *CYP6ER1vL.* The remaining two constructs, ER1vL_S and ER1vL_T_GP displayed very slight levels of resistance with RRs at LC_{50} of 1.8-fold against the *CYP6ER1vL* strain.

For nitenpyram, clothianidin, dinotefuran and thiamethoxam (Table 6.10) none of the mutant constructs showed significant resistance, with all strains having RR at LC_{50} below 2-fold against *CYP6ER1vL*. For acetamiprid ER1vL_T_GP had a RR at LC_{50} of 3.3, showing low resistance, in contrast to the other mutant constructs that did not demonstrate such resistance.

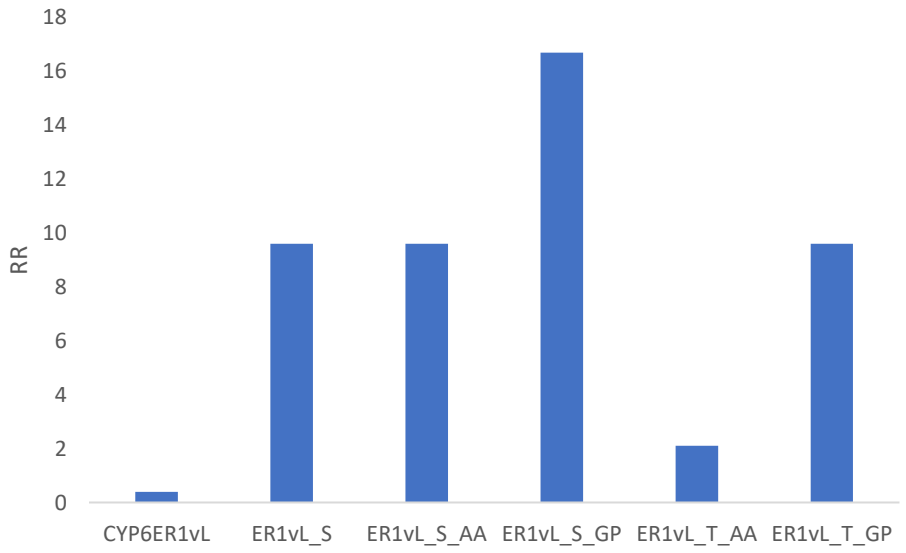


Fig. 6.15 Resistance ratios for *D. melanogaster* strains against imidacloprid

Table 6.8 Log-concentration probit mortality data for imidacloprid against *D. melanogaster* strains containing *CYP6ER1* mutants.

Compound	Strain	LC_{50} [mg/L^{-1}]	95% CL	Slope (± SD)	Resistance Ratio To Control	Resistance Ratio To CYP6ER1vL
Imidacloprid	Control	111.1	45-233	1.373 ± 0.287	1.0	2.1
	CYP6ER1vL	53.1	31.4-82.6	1.864 ± 0.328	0.4	1.0
	ER1vL_S	1062	531-2292	1.103 ± 0.183	9.6	20.0
	ER1vL_S_AA	1063	442-2680	1.54 ± 0.356	9.6	20.0
	ER1vL_S_GP	1857	905-4229	1.448 ± 0.293	16.7	35.0
	ER1vL_T_AA	237	101.4-520	1.324 ± 0.262	2.1	4.5
	ER1vL_T_GP	1062	752-1501	2.082 ± 0.264	9.6	20.0

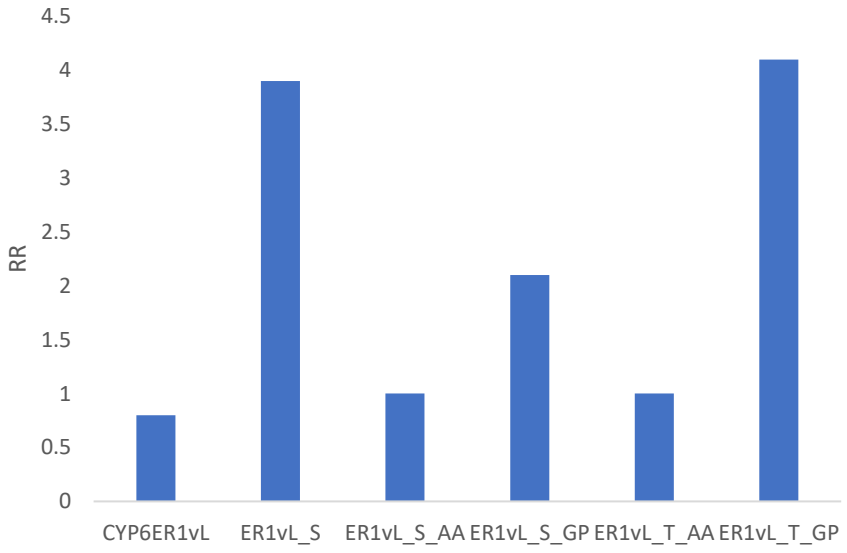


Fig 6.16 Resistance ratios for *D. melanogaster* strains against thiacloprid

Table 6.9 Log-concentration probit mortality data for thiacloprid against *D. melanogaster* strains containing *CYP6ER1* mutants.

Compound	Strain	LC_{50} [mg/L^{-1}]	95% CL	Slope (± SD)	Resistance Ratio To Control	Resistance Ratio To CYP6ER1vL
Thiacloprid	Control	198	6-1199	0.71 ± 0.248	1.0	0.4
	CYP6ER1vL	442.6	nc	6.4 ± 16.2	0.8	1.0
	ER1vL_S	781	381-1651	1.284 ± 0.227	3.9	1.8
	ER1vL_S_AA	201	132.6-302	1.642 ± 0.204	1.0	0.5
	ER1vL_S_GP	414	nc	0.754 ± 0.284	2.1	0.9
	ER1vL_T_AA	203	82-426	1.264 ± 0.224	1.0	0.5
	ER1vL_T_GP	814	348-1628	2.051 ± 0.477	4.1	1.8

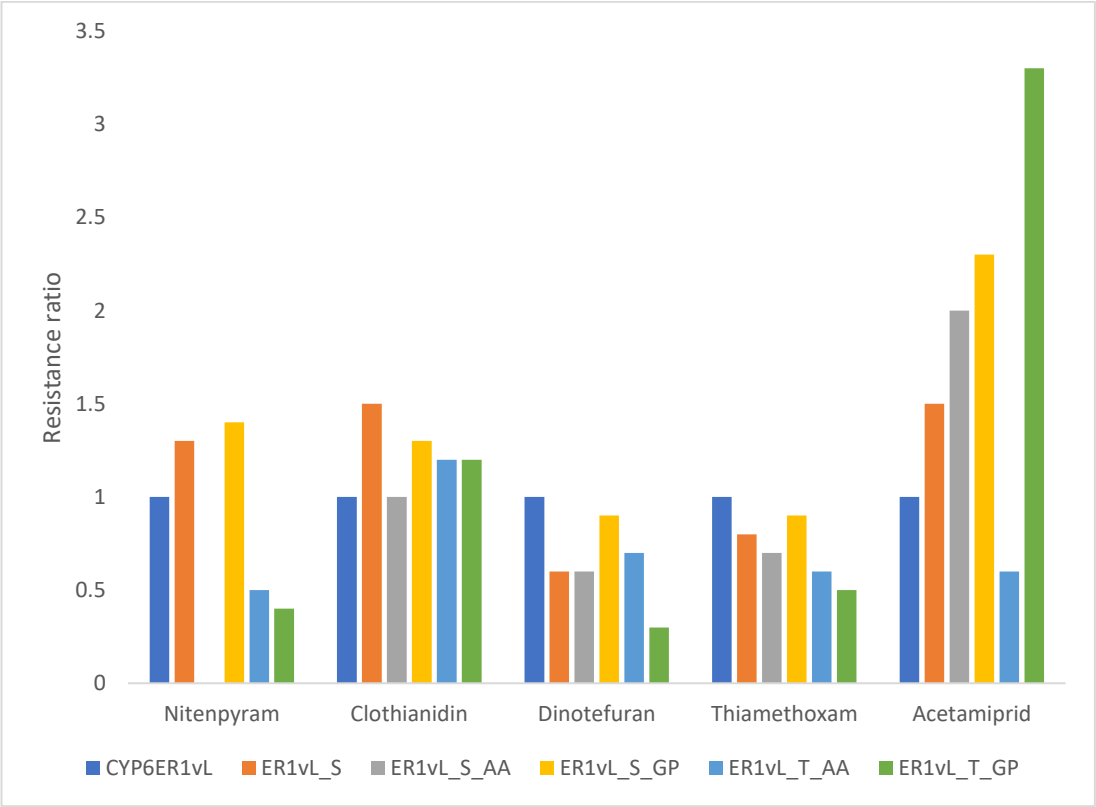


Fig. 6.17 Resistance ratios for *D. melanogaster* strains against other neonicotinoids

Table 6.10 Log-concentration probit mortality data for other neonicotinoids against *D. melanogaster* strains containing *CYP6ER1* mutants.

Compound	Strain	LC_{50} [mg/L^{-1}]	95% CL	Slope (± SD)	Resistance Ratio To Control	Resistance Ratio To CYP6ER1vL
Nitenpyram	Control	362.3	nc	12.2 ± 22	1.0	0.8
	CYP6ER1vL	446.1	370.4-537.5	5.389 ± 0.929	2.6	1.0
	ER1vL_S	575.3	471.2-694	4.566 ± 0.647	1.6	1.3
	ER1vL_S_AA	nt	nt	nt	nt	nt
	ER1vL_S_GP	632.5	582.1-687	6.452 ± 0.527	1.7	1.4
	ER1vL_T_AA	202.4	141.2-287.7	4.23 ± 0.986	0.6	0.5
	ER1vL_T_GP	176	128.7-238.6	3.612 ± 0.691	0.5	0.4
Clothianidin	Control	15.4	nc	8.5 ± 11.4	1.0	1.1
	CYP6ER1vL	14.05	nc	8.4 ± 16.2	2.8	1.0
	ER1vL_S	20.4	16.8-24.8	3.041 ± 0.322	1.3	1.5
	ER1vL_S_AA	14.6	nc	8.1 ± 13.4	0.9	1.0
	ER1vL_S_GP	18.4	nc	7 ± 10.9	1.2	1.3
	ER1vL_T_AA	16.85	12.1-23.4	3.147 ± 0.571	1.1	1.2
	ER1vL_T_GP	17.43	nc	7.22 ± 9.74	1.1	1.2
Dinotefuran	Control	34.83	23.6-52.5	3.499 ± 0.737	1.0	0.8
	CYP6ER1vL	44.72	37.4-53.5	5.247 ± 0.527	2.0	1.0
	ER1vL_S	28.39	nc	8.4 ± 11.5	0.8	0.6
	ER1vL_S_AA	24.64	nc	6.9 ± 13.7	0.7	0.6
	ER1vL_S_GP	38.17	26.3-55.4	2.76 ± 0.477	1.1	0.9
	ER1vL_T_AA	31.64	nc	9.2 ± 9.8	0.9	0.7
	ER1vL_T_GP	14.34	9-22.7	2.86 ± 0.617	0.4	0.3

	Control	18.92	nc	6.9 ± 13.6	1.0	0.6
	CYP6ER1vL	31.63	26.3-38.4	3.689 ± 0.435	3.3	1.0
	ER1vL_S	24.35	15.6-37.8	2.629 ± 0.507	1.3	0.8
Thiamethoxam	ER1vL_S_AA	21.06	nc	7.51 ± 9.56	1.1	0.7
	ER1vL_S_GP	28.74	23.6-35.2	3.444 ± 0.422	1.5	0.9
	ER1vL_T_AA	20	nc	6.6 ± 10.9	1.1	0.6
	ER1vL_T_GP	17.33	nc	6.9 ± 10.3	0.9	0.5
	Control	56	48.1-65.1	3.38 ± 0.29	1.0	1.2
	CYP6ER1vL	47.9	21.4-129	2.368 ± 0.686	0.4	1.0
	ER1vL_S	73.6	48.5-110.7	3.148 ± 0.672	1.3	1.5
Acetamiprid	ER1vL_S_AA	97.5	nc	7.6 ± 13.6	1.7	2.0
	ER1vL_S_GP	107.9	78.2-148.9	3.075 ± 0.535	1.9	2.3
	ER1vL_T_AA	28.74	23.6-35.2	3.444 ± 0.422	0.5	0.6
	ER1vL_T_GP	158.1	131.7-192.1	3.689 +0.434	2.8	3.3

The final bioassay with ethiprole (Table 6.11) showed two strains (ER1vL_S and ER1vl_S_AA) with moderate resistance to ethiprole. This was surprising since ER1vL_S_AA is most like CYP6ER1vB, which did not show significant resistance to ethiprole in the fly mortality bioassays (Table 6.6). However, *CYP6ER1vB* did demonstrate an ability to survive ethiprole in the locomotor activity assays compared to *CYP6ER1vL*, which correlates with our finding here (Table 6.11).

In contrast the construct most like *CYP6ER1vA*, ER1vL_S_GP, did not show any resistance to ethiprole, whereas *CYP6ER1vA* had shown significant resistance to ethiprole in both the fly mortality bioassay and the locomotor activity assay. Fipronil was not tested since none of the original *CYP6ER1* variants displayed any resistance against this compound.

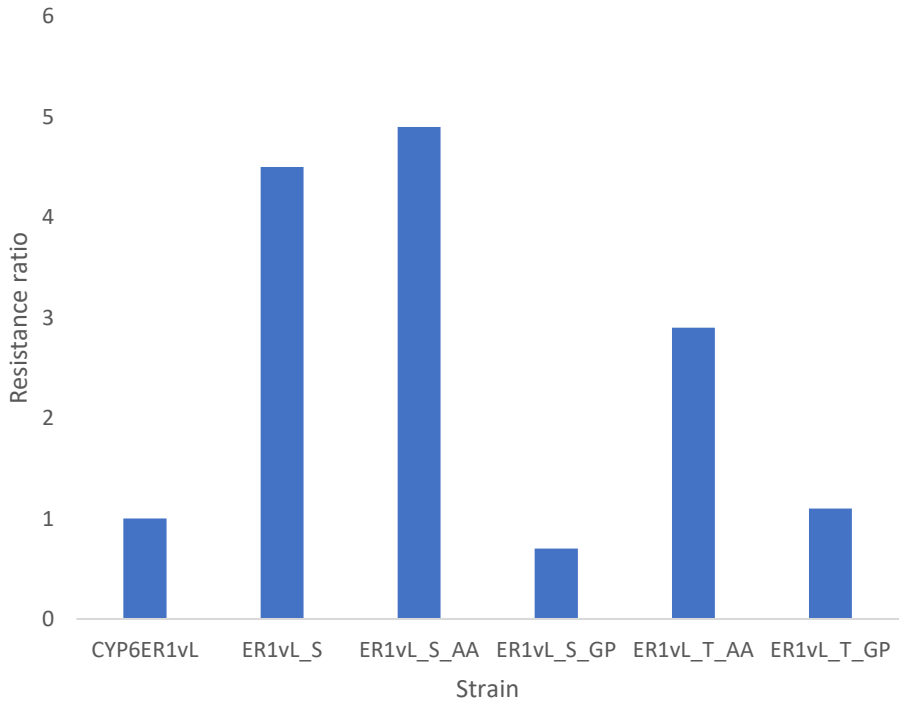


Fig. 6.18 Resistance ratios for *D. melanogaster* strains against ethiprole

Table 6.11 Log-concentration probit mortality data for ethiprole against *D. melanogaster* strains containing *CYP6ER1* mutants.

Compound	Strain	LC_{50} [mg/L^{-1}]	95% CL	Slope (± SD)	Resistance Ratio To Control	Resistance Ratio To CYP6ER1vL
Ethiprole	Control	74.8	45.4-122.9	2.103 ± 0.37	1.0	3.1
	CYP6ER1vL	23.9	11.26-42.1	1.452 ± 0.244	0.5	1.0
	ER1vL_S_AA	107.9	67.7-172	2.031 ± 0.328	1.4	4.5
	ER1vL_S	117.4	nc	7.5 ± 14.5	1.6	4.9
	ER1vL_S_GP	17.46	9.25-31.5	1.979 ± 0.418	0.2	0.7
	ER1vL_T_AA	69.5	53.8-89.9	2.317 ± 0.244	0.9	2.9
	ER1vL_T_GP	27.39	20.82-36	2.283 ± 0.254	0.4	1.1

6.3.9 *CYP4DE1* sequencing

Two cytochrome P450s were linked with ethiprole resistance in the small brown planthopper (Elzaki, Zhang and Han, 2015), and these were assessed for their potential to cause ethiprole resistance in *N. lugens*. *CYP4DE1* could be assembled using the transcriptome generated from the RNA-Seq done in this PhD. The other gene, *CYP6CW3v2* could not be found or assembled, and so no further analysis was performed on this gene. A subsequent study by Lao et al, also assembled the *CYP4DE1* gene, and submitted it to NCBI (accession no KM217042.1), (Lao *et al.*, 2015). There were no AA substitutions/deletions between the Bayer-S population and any of the ethiprole resistant populations for *CYP4DE1*, in contrast with the variation seen previously with *CYP6ER1*.

6.3.10 *CYP4DE1* qRT-PCR

qRT-PCR analysis for *CYP4DE1* was done on six *N. lugens* populations, including three populations that had undergone laboratory selection with ethiprole. The overexpression of *CYP4DE1* is shown (Fig. 6.19), using Bayer-S as the reference strain. All populations overexpressed *CYP4DE1* compared to Bayer-S, with

overexpression ranging from 4.05 to 8.52-fold. Unlike with *CYP6ER1*, where insecticide selection caused an increase in expression, this was not the case for *CYP4DE1*. The ethiprole selected populations were not significantly higher than their unselected counterparts. For two of the populations, Nl39-eth and Nl55-eth, their expression values were lower than their unselected populations, Nl39 and Nl55.

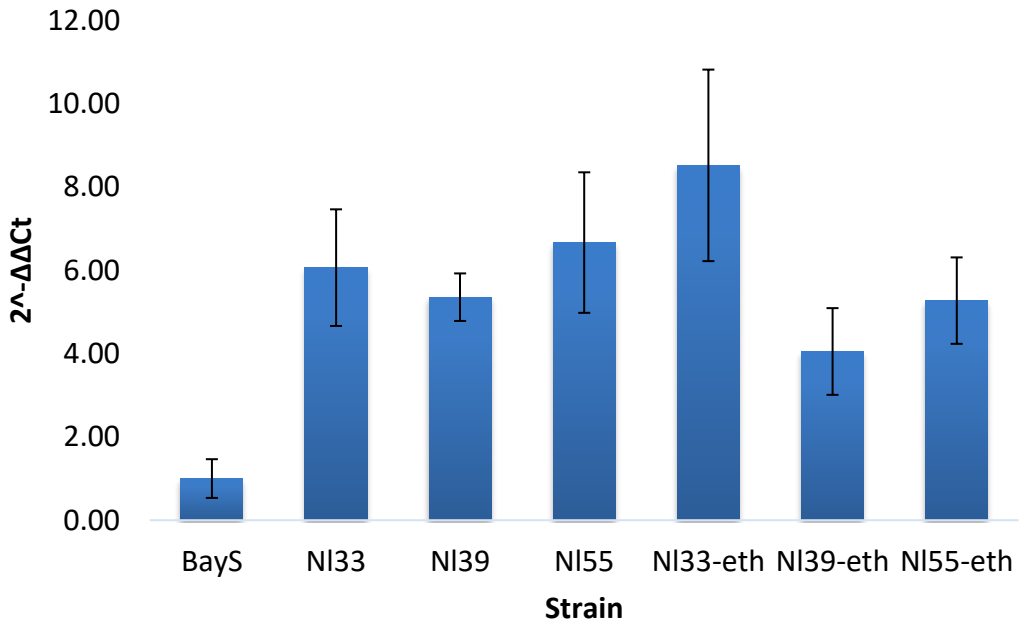


Fig. 6.19 Fold change in expression of CYP4DE1 in six ethiprole resistant *N. lugens* strains compared with the laboratory susceptible reference Bayer-S as determined by qRT-PCR. Error bars display 95% confidence intervals.

6.3.11 *CYP4DE1 D. melanogaster* bioassays

The *CYP4DE1* gene was injected into *D. melanogaster* using the same methodology as described previously. The strain created was then tested against two fiproles and two neonicotinoids (Table 6.12). For ethiprole there was high resistance displayed, with a RR at LC_{50} of 4.37. As with *CYP6ER1vA*, the high resistance to ethiprole did not coincide with resistance to fipronil. It was not possible to calculate

an LC_{50} for fipronil, since there was full mortality at all doses tested. For the neonicotinoid testing, the response to imidacloprid and thiacloprid was similar with RRs at LC_{50} of ~2-fold. So *CYP4DE1* displayed slight resistance to these two neonicotinoids, but was below that seen by *CYP6ER1vA* and *CYP6ER1vB*.

Table 6.12 Log-dose probit mortality data for neonicotinoids and fiproles against *D. melanogaster* strains .

Compound	Strain	LC_{50} [mg/L^{-1}]	95% CL	Slope (± SD)	Resistance ratio
Ethiprole	Control	89	51.6-153	1.491 ± 0.219	1.0
	CYP4DE1	389	247.1-616	2.276 ± 0.403	4.4
Fipronil	Control	2.722	2.38-3.03	5.648 ± 0.452	1.0
	CYP4DE1	Nc	nc	nc	nc
Imidacloprid	Control	445	267-739	1.473 ± 0.202	1.0
	CYP4DE1	884	549-1445	1.405 ± 0.18	2.0
Thiacloprid	Control	321	88-828	1.117 ± 0.269	1.0
	CYP4DE1	598	219-1675	1.011 ± 0.216	1.9

6.4 Discussion

The widespread use of imidacloprid since the mid-1990s as a control agent for *N. lugens* has led to high levels of resistance being seen across Asia. Given that resistance was first seen in 2003 (Matsumura *et al.*, 2008), many mechanisms of resistance to imidacloprid have since been proposed. The aim of this chapter was to evaluate the numerous proposed mechanisms of resistance to imidacloprid, and see if any of these mechanisms provided cross-resistance to other neonicotinoids. The metabolic mechanisms were also assessed for potential cross-resistance to fiproles, and a P450 previously linked with ethiprole resistance was also assessed.

The enhanced production of cytochrome P450 monooxygenases is a common route of resistance to neonicotinoids in insect pests (Karunker *et al.*, 2008; Puinean, Foster, *et al.*, 2010), and resistance to neonicotinoids in *N. lugens* has long been linked to enhanced detoxification mediated by P450s (Liu *et al.*, 2005; Nauen and Denholm, 2005; Wen *et al.*, 2009). In *N. lugens* two P450 enzymes have been linked to imidacloprid resistance through over-expression; *CYP6ER1* (Bass, Carvalho, *et al.*, 2011) and *CYP6AY1* (Ding *et al.*, 2013). Of the 12 resistant populations tested for these two genes I found that *CYP6ER1* was significantly overexpressed in all of them, compared to the susceptible reference strain. Whereas, *CYP6AY1* was under expressed in ten of the strains and showed low overexpression (3.5-fold) in only one strain (NI59). The increase in *CYP6ER1* expression when a population was selected with imidacloprid provided further evidence that this gene was involved in imidacloprid resistance. Another notable point was that variation in the biological replicates for *CYP6ER1* decreased after selection with imidacloprid (shown by the reduced 95% confidence limits). This suggested that selection with imidacloprid reduced the genetic heterogeneity within these two selected populations, and that the CYP was being expressed highly in all replicates. Such an increase in expression was not seen for *CYP6AY1*, with the imidacloprid selected populations still showing under expression of this CYP compared to the susceptible population. There was also significant variation in expression of this CYP across biological replicates.

These findings further reinforced the view that *CYP6ER1* was the major P450 mediating imidacloprid resistance in the *N. lugens* populations I studied. The lack of expression of *CYP6AY1* was surprising given the findings in the original study (Ding *et*

al., 2013), so further qRT-PCR was performed using the primers designed in the original study. Again, results correlated with our initial findings, that this CYP was under expressed compared to the susceptible. The study that implicated *CYP6AY1* had used a strain selected in the laboratory for 40 generations, and provided no comparison to an unselected parental line of the resistant strain. The populations that Ding et al saw the overexpression (4-9-fold) of *CYP6AY1* in were all from China. It is therefore possible that this CYP is overexpressed in populations from China, but not from the rest of Asia. We have been unable to obtain any field samples from China to test since the implication of the Nagoya protocol (effective October 2014), and so are unable to verify this. However, we had strong evidence that over expression of *CYP6ER1* contributed to imidacloprid resistance in *N. lugens*, and a logical next step was to functionally validate this CYP's ability to detoxify imidacloprid.

In the original study on *CYP6ER1* (Bass, Carvalho, *et al.*, 2011), it was noted that there were several polymorphisms in the coding sequence of *CYP6ER1* between the imidacloprid resistant strains and the susceptible strain. These highlighted four conserved AA substitutions in these resistant strains, and discussed that further work was necessary to determine if these changes could have functional significance in how *CYP6ER1* detoxifies imidacloprid. Therefore, we sequenced *CYP6ER1* in a range of *N. lugens* populations to see if there was further differentiation in the coding sequences of *CYP6ER1* across the resistant populations and the susceptible. This revealed that there was significant variation, even more extensive than seen in the original study, with eight *CYP6ER1* variants being present in the populations. A study

reported in 2015 detailed the role that allelic variation of P450s could play in insecticide resistance (Ibrahim *et al.*, 2015). Two genes, *CYP6P9a* and *CYP6P9b*, were found to have variations in the coding sequence between pyrethroid resistant populations and a susceptible population of *Anopheles funestus*. The P450s in the resistant strains were shown to be undergoing directional selection and had accumulated mutations that proved beneficial to pyrethroid metabolism. Multiple lines of evidence were provided including metabolism assays, modelling and docking simulations and transgenic expression in *D. melanogaster* which, taken together, demonstrated that the observed allelic variation was a key driver of pyrethroid resistance (Ibrahim *et al.*, 2015). Seeing such significant variation in the *CYP6ER1* gene across the *N. lugens* populations led to the hypothesis that allelic variation could play a role in imidacloprid resistance in a similar way to the study by Ibrahim *et al* above. The extent to which certain alleles were selectively expressed over others and the role of quantitative vs qualitative in *CYP6ER1* comprised most of the analysis of metabolic resistance in *N. lugens*.

Since it seemed implausible that all these variants were being overexpressed it was decided to perform variant specific qRT-PCR to determine which were the key *CYP6ER1* variants. This clearly demonstrated that there were two key *CYP6ER1* variants that were highly expressed in insecticide resistant field populations of *N. lugens*: *CYP6ER1vA* and *CYP6ER1vB*. These are never significantly expressed together within a strain and it appears that the variant expressed depends on geographical location. The *CYP6ER1vB* variant is only ever seen in India, whilst *CYP6ER1vA* is seen across Southeast Asia. Both variants differ from the other sequenced variants in that

they have a profound polymorphism – a 3bp indel that results in the loss of an amino acid in SRS-5.

To establish the extent to which different natural *CYP6ER1* variants could provide resistance to neonicotinoids, several variants were transformed into *D. melanogaster*. *CYP6AY1* was also included in this analysis to see whether it could metabolise imidacloprid, despite its low expression levels in resistant *N. lugens*. This showed that two fly strains that overexpressed *CYP6ER1vA* and *CYP6ER1vB* respectively, were significantly more resistant than a control fly strain when tested with imidacloprid and the CYP6ER1 variant seen in the susceptible strain of *N. lugens* - which conferred no resistance in flies. This correlated with the fact that these were the two *CYP6ER1* variants highly expressed in resistant *N. lugens* populations, and demonstrated unequivocally the ability of *CYP6ER1* to confer imidacloprid resistance. A recent study also found that transgenic expression of *CYP6ER1* in *D. melanogaster* caused significant resistance to imidacloprid (Pang *et al.*, 2016). Although not discussed by the authors this study appears to use a *CYP6ER1* variant that is like the *CYP6ER1vA* seen in our *N. lugens* imidacloprid resistant strains, and came from a strain captured from Guangxi, China.

Issues were seen with the *D. melanogaster* insecticide bioassays in that the control strain always showed significant resistance to imidacloprid, meaning very high doses of insecticide were necessary to obtain LC_{50}s and 95% confidence limits. This is likely due to *CYP6G1*, a P450 naturally expressed in *D. melanogaster* that provides resistance to a range of insecticides including imidacloprid (Daborn, J. L Yen, *et al.*, 2002). However, we also found that mortality of a fly strain could be

significantly lower than that of the control strain, for example, a strain expressing *CYP6ER1vL* is much less resistant to imidacloprid than the control strain. So, it appears that expressing a transgene that does not contribute to resistance causes a significant fitness cost in that fly strain. Comparing resistant fly strains to the strain expressing *CYP6ER1vL* shows an even higher level of resistance demonstrated by strains expressing *CYP6ER1vA* and *CYP6ER1vB*. To strengthen the *D. melanogaster* insecticide bioassay results, a different bioassay and locomotor activity monitoring were performed to see whether *CYP6ER1vA* conferred resistance in these assays. The wiggle index bioassays again showed that *CYP6ER1vA* could provide significant resistant compared to a strain expressing *CYP6ER1vL*. The locomotor activity assays are more complex to interpret and do not yield easily comparable statistics, like RRs. However, the overall profile of the locomotor activity assay with imidacloprid showed that the *CYP6ER1vA* strain was the least disrupted by exposure to imidacloprid. These bioassays provide convincing evidence that changes in the coding sequence of *CYP6ER1* was also a significant part of resistance to imidacloprid, rather than just overexpression of the gene.

Interestingly the fly strain expressing *CYP6ER1vA* was also able to provide significant resistance to ethiprole, suggesting this enzyme can provide cross-resistance. Instances of P450s providing cross resistance to insecticides involving neonicotinoids have been noted before in *D. melanogaster* (Daborn *et al.*, 2001) and *M. persicae* (Bass *et al.*, 2014). However, whether *CYP6ER1vA* contributes a significant role in ethiprole resistance in *N. lugens* populations is somewhat unclear. There does appear to be an increase in expression of *CYP6ER1vA* in strains that are

selected with ethiprole, however the Nl55-eth strain (which is highly resistant to ethiprole) carries *CYP6ER1vB* instead, and this variant showed no resistance in fly mortality bioassays with ethiprole. Surprisingly, the locomotor activity assays demonstrated that *CYP6ER1vB* could potentially provide minor ethiprole resistance. However, the unexplained fipronil mechanism is a more likely candidate in ethiprole resistance, but it is still possible that *CYP6ER1vA/CYP6ER1vB* can contribute towards ethiprole resistance, though if so it would only be in a minor capacity. This is partly because the LC_{50} for ethiprole in Nl39-imi (23.27) is significantly less than that for Nl39-eth (>5000). If *CYP6ER1vA* was a significant driver of ethiprole resistance in *N. lugens*, I would have expected the LC_{50} from the ethiprole bioassay for Nl39-imi to be considerably higher.

Given we now knew which were the key *CYP6ER1* variants, we wanted to isolate which were the key changes in the coding sequence that contributed to imidacloprid resistance. Mapping of substrate recognition sites revealed the deletion at position 377 and the resulting AA changes occur in SRS-5. Furthermore, a second threonine to serine replacement was also observed in SRS-4 within the oxygen binding motif.

Threonine is generally substituted by polar or small amino acids, especially serine due to similarity between the two amino acids. A substitution of Ser for Thr could cause more flexibility in the conformations that the main chain can adopt, since Thr is C-beta branched and has restrictions on structural conformation (Betts and Russell, 2003). The deletion of an alanine and further substitution of an alanine for a glycine (PAAP to PGP) would have considerable impact on protein structure. Gly

allows much more conformational flexibility, allowing tight turns in structures, that other amino acids find impossible to adopt (Betts and Russell, 2003). The deletion of a proline (PAAP to PAA) could prevent the polypeptide from changing direction, since Pro is unable to adopt many main chain conformations (due to ring structure) and so is usually found in tight turns of protein structure (Betts and Russell, 2003).

Constructs that encoded the *CYP6ER1vL* sequence with AA changes around position 377, to make the sequence more like *CYP6ER1vA*/*CYP6ER1vB*, were tested using the *D. melanogaster* insecticide bioassays. This identified that the change from PAAP to PGP (deletion and substitution), provided the most resistance to imidacloprid. PGP is seen in the *CYP6ER1vA* variant. The crucial *CYP6ER1vB* change (deletion of 377P) also conferred resistance, but not as high as the GP change. A further key AA substitution was seen at T318S, with the serine replacement also able to confer significant resistance to imidacloprid.

Given the abundance of examples demonstrating that overexpression of P450s causes resistance to insecticides there are comparatively few examples available showing qualitative changes in P450s correlating with resistance. A CYP from *D. melanogaster* (*CYP6A2*) has been linked to DDT resistance in a strain ($RDDT^R$) (Amichot *et al.*, 2004). There three point mutations (R335S, L336V and V476L) within *CYP6A2* were correlated with enhanced metabolism of DDT compared to a wild-type version of *CYP6A2*. A more recent example was discussed above involving allelic variation in CYPs causing pyrethroid resistance in *A. funestans* (Ibrahim *et al.*, 2015). Multitudes of described examples are available for human P450s, with extraordinary diversity seen within this gene super family. For example, *CYP2C9* has a recorded 33

variants and further series of subvariants (Zhou, Liu and Chowbay, 2009). Within this there have been 520 SNPs isolated from the upstream, intron and exonic sequence. Different variants have different levels of activity and ability to metabolise xenobiotics depending on which SNPs are present in regulatory and coding regions (Zhou, Liu and Chowbay, 2009).

Research conducted by colleagues at Exeter university has revealed a fuller picture of the *CYP6ER1* gene, and has concluded that the gene has been duplicated in the neonicotinoid resistant populations of *N. lugens*. This duplication occurred before the introduction of imidacloprid for field control, so the duplication is not a novel mutation occurring due to insecticide use. However, the additional copy was not evolutionarily constrained and so was able to accrue mutations that were not possible in single copy populations. These mutations caused the enzyme to become an effective metaboliser of imidacloprid and so caused resistance to this compound.

Another P450, *CYP4DE1*, was also assessed for a contribution to insecticide resistance in *N. lugens*. This gene was originally linked to ethiprole resistance in the small brown planthopper through overexpression of this CYP (Elzaki, Zhang and Han, 2015). Studies using qRT-PCR on ethiprole resistant *N. lugens* populations did show over expression (4-9-fold) of this gene compared to the susceptible. However, the fold change is significantly lower than that seen for *CYP6ER1*, as a comparison, and selection with ethiprole had no change in the expression of this gene. When *CYP4DE1* was expressed in a fly strain it did cause significant resistance to ethiprole (4-fold) compared to the control strain. However, as with *CYP6ER1vA* and ethiprole

resistance, it is unclear whether *CYP4DE1* has a biologically significant role in ethiprole resistance within *N. lugens*.

6.5 Conclusions

This study aimed to evaluate the potential metabolic mechanisms behind neonicotinoid and fiprole resistance. This involved assessing expression levels of multiple P450s across a range of *N. lugens* populations. The P450s were all then functionally validated to provide evidence of their potential role in detoxification of insecticidal compounds.

CYP6ER1 was implicated as a major contributor to imidacloprid resistance, rather than other proposed CYPs and target-site mutations/down-regulation. Crucial changes in the coding sequence of *CYP6ER1* were identified that confer an ability to metabolise imidacloprid. This gene was extensively studied to understand why it could be such a potent mechanism in imidacloprid resistance.

Chapter VII General Discussion

This chapter provides a general discussion and summary of the findings of this PhD. The resistance to phenylpyrazole and neonicotinoid compounds is discussed and the implications that this has for field management of *N. lugens* is briefly touched upon. Potential future work that could be conducted in this area is also highlighted.

7.1 Fiprole resistance in *N. lugens*

Prior to the start of this PhD, high levels of resistance to ethiprole and fipronil had been reported in various *N. lugens* field populations (Wang *et al.*, 2009; Zhao *et al.*, 2011; Punyawattoe *et al.*, 2013) collected in the Asia Pacific region. In Chapter III, *N. lugens* populations sourced from across Asia were tested for fiprole resistance and high levels of resistance were found in all the field strains examined, correlating with the results of previous monitoring studies. Further selection of a population in the laboratory with ethiprole caused the resistance to ethiprole to greatly increase. However, it also caused the levels of resistance to fipronil to markedly increase. This was seen for three separate *N. lugens* populations, and gave weight to the hypothesis that there could be a cross-resistance mechanism between these two compounds. The structural similarity between ethiprole and fipronil (Caboni, Sammelson and Casida, 2003) further reinforced the notion of a cross-resistance mechanism.

Within the field populations of *N. lugens* held at Rothamsted it was discovered that in some individual brown planthoppers there were mutations present within the *Rdl* gene, which encodes the GABA-gated chloride channel that harbour's the fiprole binding site, that could be conferring resistance to fiproles. These included a common mutation, A301S, that was originally identified as being

responsible for high levels of resistance to dieldrin in *D. melanogaster* (ffrench-Constant *et al.*, 1990, 1993). This mutation has also been tentatively linked to fipronil cross-resistance in *N. lugens* (Y Zhang *et al.*, 2016). A further (novel) mutation, Q359E, was identified within an Indian field population. This novel mutation was not situated within the transmembrane region of the GABA-gated chloride channel, but rather in a region that had previously been linked with fipronil resistance. An adjacent R357Q mutation (in tandem with A301N) was reported to cause fipronil resistance in *S. furcifera* (Nakao *et al.*, 2012), whilst a T360I mutation (in tandem with A301S) caused fipronil resistance in *D. melanogaster* (Remnant *et al.*, 2014).

The A301S mutation became fixed in populations that were further selected with ethiprole in the laboratory. The Q359E mutation also increased in frequency after the selection with ethiprole of the NI55-eth population, originally sourced from India. However, only the A301S mutation proved to have a significant link to ethiprole resistance from the *in vivo* data collected in this PhD. It was interesting that this mutation had not previously been linked to ethiprole resistance. However, this is likely due to the fact that there has been limited research published on ethiprole resistance, and these previous studies tended to conclude that the mechanism was probably metabolic rather than target-site resistance (Punyawattoe *et al.*, 2013; Elzaki, Zhang and Han, 2015). In this thesis, despite the very high levels of ethiprole resistance seen in a fly strain carrying the A301S mutation (sourced from the Bloomington collection), it was thought that this mutation could not be the only mechanism behind ethiprole resistance. This was based on the *in vitro* electrophysiological studies conducted by Bayer CropScience on this mutation. These concluded that the difference between the A301S and the wild-type RDL constructs

in the voltage clamp recordings, with ethiprole, could not completely explain the resistance seen in the selected strains (Nl33-eth, Nl39-eth and Nl55-eth) (Garrood *et al.*, 2017). This led to the hypothesis that the undiscovered fipronil mechanism could potentially cause cross-resistance between the phenylpyrazoles compounds fipronil and ethiprole.

The possibility of metabolic mechanisms being involved in fiprole resistance was explored. There was no significant effect on fipronil resistance in *N. lugens* when synergists that disrupt P450s were applied, suggesting that P450s don't contribute to fipronil resistance. Analysis of the *CYP6ER1* variants that were assessed for a possible role in neonicotinoid resistance (Chapter VI) demonstrated that two of these variants (*CYP6ER1vA* and *CYP6ER1vB*) could cause resistance to ethiprole. These were also the variants that were implicated in imidacloprid resistance in *N. lugens*. Whether there is genuine biological significance (i.e. can cause ethiprole resistance in *N. lugens*) for the finding that these two variants can cause ethiprole resistance when expressed in *D. melanogaster* needs further analysis. The lack of resistance shown to fipronil by *D. melanogaster* strains expressing *CYP6ER1vA* or *CYP6ER1vB* further highlights the difference in response to the two phenylpyrazoles, despite their structural similarity (Caboni, Sammelson and Casida, 2003).

Cross-resistance within an insecticide class has been shown to be relatively common (Gorman *et al.*, 2010). Indeed, the field strains studied in this thesis displayed resistance to both ethiprole and fipronil, but it became clear that there was very limited cross-resistance associated with the main ethiprole resistance mechanism (A301S). However, it is rarer to see cross-resistance occur between

distinct classes, where the chemical structures will be considerably different. Examples of this include *CYP6G1* causing resistance to DDT and imidacloprid in *D. melanogaster* (Daborn *et al.*, 2001), and a P450, *CYP6CM1* causing cross-resistance between pymetrozine and neonicotinoids in *B. tabaci* (Gorman *et al.*, 2010; Nauen *et al.*, 2013). Whether *CYP6ER1vA* and/or *CYP6ER1vB* do the same between ethiprole and imidacloprid would need further studies.

7.2 Neonicotinoid resistance in *N. lugens*

Target-site resistance to neonicotinoids has previously been reported, with mutations in nAChR linked to a reduced sensitivity to neonicotinoids. These include a Y151S mutation in an nAChr α1 subunit found in *N. lugens* (Liu *et al.*, 2005), and an R81T mutation in the nAChR β subunit of *M. persicae* (Bass, Puinean, *et al.*, 2011). However, in this thesis, screening of the *N. lugens* nAChR subunits in imidacloprid resistant brown planthoppers (Chapter IV, using the RNA-seq mapping technique), reveal that neither of these mutations were present in our *N. lugens* populations. This correlated with previous research on neonicotinoid resistance in *N. lugens*, that was unable to identify the Y151S mutation (Gorman *et al.*, 2008; Puinean, Denholm, *et al.*, 2010).

The role of cytochrome P450 monooxygenases in neonicotinoid resistance has been previously well established across many insect pests. For *N. lugens* there was significant evidence that overexpression of P450s was the mechanism behind field resistance to imidacloprid (Liu *et al.*, 2003; Wang *et al.*, 2009). Of the two P450s (*CYP6ER1* and *CYP6AY1*) (Bass, Carvalho, *et al.*, 2011; Ding *et al.*, 2013) most prominently linked to imidacloprid resistance in *N. lugens*, it was found that only

CYP6ER1 was significantly overexpressed in the field populations held at Rothamsted. The original study of *CYP6AY1* (Ding *et al.*, 2013) did not include *CYP6ER1* in the qRT-PCR studies. Also, all strains assessed in that study were from China, whilst our *N. lugens* collection did not include populations from this region, so there was a possibility that *CYP6AY1* was only overexpressed in Chinese populations, whilst *CYP6ER1* was overexpressed in populations from across the rest of Asia. However, subsequent analysis of imidacloprid resistant Chinese populations has revealed that *CYP6ER1* is indeed overexpressed in these populations (Bao *et al.*, 2015; Pang *et al.*, 2016; Yixi Zhang *et al.*, 2016). These studies have all provided ample evidence that *CYP6ER1* performs a key role in imidacloprid resistance in *N. lugens*. However, there has been no analysis conducted into whether coding sequence variations between imidacloprid susceptible and resistant insects has a contribution to this well-established resistance.

This PhD follows on from the original work conducted on *CYP6ER1*, that highlighted that there was coding sequence variation between the laboratory susceptible and the field resistant *N. lugens* populations (Bass, Carvalho, *et al.*, 2011). At the time the conclusions drawn were that overexpression of *CYP6ER1* was the main mechanism responsible for imidacloprid resistance. However, it is now clear that there is a much more complex picture of resistance involving this enzyme. The sequencing of this gene across various *N. lugens* populations revealed that there were eight variants of *CYP6ER1* present. Of these, two (*CYP6ER1vA* and *CYP6ER1vB*) were found to be the dominantly expressed variants, demonstrated by variant specific qRT-PCR studies and by aligning RNA-Seq reads to specific fragments of sequence. These two variants were unique in that they both contained a mutation in

the SRS-5 region of *CYP6ER1* and a further substitution in SRS-4. These mutations occur in regions predicted to be responsible for binding of imidacloprid, as shown for *CYP6CM1vQ* in *B. tabaci* (Karunker *et al.*, 2009). Of the five *CYP6ER1* variants expressed in *D. melanogaster*, these two variants conferred the most significant resistance to imidacloprid. The *CYP6ER1vL* variant from the susceptible *N. lugens* strain was considerably less resistant to imidacloprid, even more so than the control *D. melanogaster* strain used in the fly studies. This provides clear evidence that it is the coding sequence alterations in *CYP6ER1* that has enabled this enzyme to confer imidacloprid resistance, rather than just overexpression as had been previously reported (Bass, Carvalho, *et al.*, 2011; Garrood *et al.*, 2016). Furthermore, when the mutations in SRS-4 and SRS-5 were introduced into *CYP6ER1vL* and the modified P450 subsequently expressed in *D. melanogaster,* there was a highly significant resistance displayed to imidacloprid compared to the *CYP6ER1vL* expressing *D. melanogaster* strain. This identification of key amino acid changes in P450 enzymes provides a rare example of qualitative, rather than quantitative metabolic resistance to an insecticide.

7.3 The problems for insecticide resistance management of *N. lugens*

The key findings of this PhD are that, in the *N. lugens* populations studied, A301S causes significant resistance to ethiprole, whilst certain variants of *CYP6ER1* cause resistance to imidacloprid.

The A301S mutation, conferring resistance to ethiprole, can be simply screened for in field populations, as the monitoring can be conducted on DNA, which allows for a more high-throughput molecular diagnostic than RNA. It also allows

simpler transportation and storage of the insects than that required for material that is destined for RNA studies. A similar approach could be taken for *CYP6ER1* imidacloprid resistance monitoring. Current dogma is that overexpression of this gene is the key reason for imidacloprid resistance. However, we have found that qualitative changes in the coding sequence are more important to resistance than overexpression. Therefore, a molecular diagnostic could be designed that screens for the deletion in SRS-5, and this could be performed on DNA.

Current guidelines for insecticide resistance management for rice hoppers are based on the assumption that there is no cross-resistance between insecticides used for control (IRAC, 2013). The findings of this PhD have important implications for resistance management strategies for *N. lugens*. Firstly, there is clear evidence that selection with ethiprole also causes a rise in fipronil resistance. Therefore, regions that are seeing field control failure with ethiprole should not be treated with fipronil, since it will likely be ineffective for control of *N. lugens*. A possible mechanism of cross-resistance between imidacloprid (Class 4 insecticide) and ethiprole (Class 2B insecticide) is seen in the form of a P450, *CYP6ER1*. There is a slim possibility that a *N. lugens* field population that was resistant to imidacloprid could then subsequently be resistant to ethiprole. However, the use of imidacloprid would not lead to a rise in fipronil resistance. It would also depend on whether *CYP6ER1* was responsible for the imidacloprid resistance present, since multiple P450s have now been implicated in imidacloprid resistance in *N. lugens* (Yixi Zhang *et al.*, 2016). The studies on *CYP6ER1* in this PhD indicate that it is not able to cause widespread resistance across the neonicotinoid class of insecticides. However, there is now resistance to most of the neonicotinoids being reported in the field (Matsumura *et al.*, 2013; Zhang *et al.*,

2014, 2017; X. Zhang *et al.*, 2015; Mu *et al.*, 2016) and the mechanisms behind this resistance are not yet clear. There is no reported cross-resistance between imidacloprid and pymetrozine (Class 9 insecticide), another important chemical for *N. lugens* control (Yang *et al.*, 2016).

Given that resistance to ethiprole and imidacloprid is now so well established within *N. lugens* field populations (Garrood *et al.*, 2016), it is debatable if there will come a point in the future where these compounds will once again become viable control agents for *N. lugens*. A large determining factor will be whether the mechanisms associated with the resistance carry a fitness cost in the absence of the xenobiotic. In the case of ethiprole resistance it is unlikely that the A301S mutation has a significant fitness cost, since this mutation has been shown to be retained in a pest population even in the absence of insecticide selection (Thompson, Steichen and ffrench-Constant, 1993). It would normally be expected that P450 mediated resistance would carry a fitness cost in the absence of insecticide selection, however this could potentially not be the case with *CYP6ER1*. Firstly, *N. lugens* populations kept in the laboratory remained resistant to imidacloprid long after the last exposure to imidacloprid. However, predicting fitness costs effects in the field from laboratory populations is fraught with pitfalls, since a variety of other factors (temperature fluctuations, predation, intra-species competition, exposure to other chemistries and host plant variety) cannot be considered. Secondly, given that *CYP6ER1* mediated resistance is predominantly due to changes in the coding sequence that have caused certain variants (*CYP6ER1vA* and *CYP6ER1vB*) to become highly efficient metabolisers of imidacloprid, rather than overexpression, this could reduce the associated fitness cost.

A new compound for *N. lugens* control has recently become available called triflumezopyrim, and belongs to group 4E of IRAC's MoA classification system (Singh *et al.*, 2016). This was developed by DuPont Crop Protection and is very effective against *N. lugens*, even those with high imidacloprid resistance (Cordova *et al.*, 2016). This is currently the only new compound being registered for *N. lugens* control. However, the manufacturers hope that since triflumezopyrim should provide long lasting control, then less applications will be necessary. This would benefit natural enemies and so improve the rice ecology that has been seriously disrupted by heavy insecticide use (Singh *et al.*, 2016).

Integrated Pest Management strategies for rice have been designed for decades, with a book produced on the topic in the 1980s (Reissig *et al.*, 1988) The basis of IPM for rice now emphasises the importance of allowing natural enemies to flourish, primarily through a reduction in insecticide use (Matteson, 2000). This strategy is now favoured, in part, because most compounds for *N. lugens* control are now unable to give the requisite level of control that farmers desire. IRRI specifically state that indiscriminate use of insecticides must be avoided and that mechanical and biological methods should be the primary strategy for managing the brown planthopper (IRRI, 2017). The use of rice cultivars that are resistance to *N. lugens* should also be used (Brar *et al.*, 2009; IRRI, 2017). Way and Heong discuss a system whereby multiple cultivars with different resistant mechanisms to pests are used, within a patchwork of fields to prevent build-up of pest resistance to these cultivars (Way and Heong, 1994). A more recent appraisal of the brown planthopper problems and methods to control it our provided by Bottrell and Schoenly. Of their many suggestions for *N. lugens* control that do not rely on heavy use of pesticides, the need

for an Asia-wide effort to formulate an effective strategy with long-term sustainability is emphasised (Bottrell and Schoenly, 2012). Such a strategy would be a huge undertaking, but it is necessary, given that the evidence has for decades shown that insecticide use cannot fully control *N. lugens* and has been argued exacerbates the problem. Given that a point has now been reached where the arsenal of compounds used to control *N. lugens* has dwindled to only a few, there must be a concerted effort driven to limit the effect of this destructive pest.

7.4 Future work

1. The unidentified mechanism behind fipronil resistance in *N. lugens* could be further explored. Synergist bioassays have suggested that this mechanism in unlikely to be P450 related, and there are no mutations in *Rdl* that correlate with significant resistance to fipronil. Further synergists could be tested to establish if CCEs/GSTs have any potential role in this resistance. Penetration resistance could be investigated to see whether a reduced uptake of fipronil contributes to fipronil resistance. Any mechanism discovered that links to fipronil resistance would also need to be tested against ethiprole, to test the hypothesis that the unexplained fipronil resistance mechanism confers cross-resistance to ethiprole.
2. The transcriptome generated in Chapter IV could be complemented by the generation of a transcriptome using genome guided assembly. This would significantly improve the gene annotation and would allow a more meaningful differential gene expression analyses to be conducted. This would

provide a useful resource to help identify the unexplained fipronil resistance mechanism.

3. The RDL mutation, Q359E, was not fully analysed within this PhD. Further studies could be conducted using CRISPR/*Cas9* to introduce the mutation into *D. melanogaster*. A fly strain containing both A301S and Q359E should ideally be generated, since the Q359E mutation never occurs independently. Fly mortality bioassays with fipronil and ethiprole should confirm whether the Q359E mutation can confer any resistance to phenylpyrazoles compounds.
4. Further studies on *CYP6ER1* variants and ethiprole resistance could be performed. The new climbing assay for *D. melanogaster* at Rothamsted could be used to see if certain *CYP6ER1* variants can provide significant levels of resistance to ethiprole.
5. Low resistance to pymetrozine in field populations of *N. lugens* has recently been reported. Although resistance is hard to quantify for this compound (due to different bioassay methods used in the monitoring efforts) it would be interesting to study field strains that had been linked to control failure using pymetrozine. A starting point would be analysing the TRP gene in the field strain for the presence of novel mutations, by comparing to the TRP gene in older field strains (not exposed to pymetrozine) and the laboratory susceptible strain.

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Appendices

Appendix A Primer sequences and qRT-PCR melt peaks

Table A1. Cloning primers and CRISPR gRNAs.

Primer name	Sequence 5'-3'
M13(-20)_F	GTAAAACGACGGCCAGT
M13(-24)_R	AACAGCTATGACCATG
T7_F	TAATACGACTCACTATAGG
T3_R	AATTAACCCTCACTAAAGGG
Dm_301_gRNA_F	GTCGCAATGCAACGCCGGCGCGTG
Dm_301_gRNA_R	AAACCACGCGCCGGCGTTGCATTG
Dm_359_gRNA_F	GTCGATACGCCACGGTCGGCTACA
Dm_359_gRNA_R	AAACTGTAGCCGACCGTGGCGTAT

Table A2. RNA-Seq candidate genes qRT-PCR primers.

Primer name	Sequence 5'-3'
c91313_g1_i1 F	AATCGTGGGCAGGAGGAGAT
c91313_g1_i1 R	TGCAACCTTCCCTTTTTCGATG
c91960_g1_i2 F	CACCAACACATCGGCGTAG
c91960_g1_i2 R	GTATGTGAGATGGGATGGACA
c98716_g1_i1 F	CCTCCTTCTCTCTCAGTTTGTG
c98716_g1_i1 R	GAAGAATTGGTGACTCGATGC
c103516_g1_i4 F	CTAGAGTATCAGTATCGTGGGTA
c103516_g1_i4 R	CAGGCGATAGCGGACAAATAC
c103516_g1_i6 F	GTGGAGCATAAGTACCGTGAG
c103516_g1_i6 R	TCATGAAACCGAGGGGTCC
c103740_g1_i2 F	CCATCGGATTACATATTGTTTGG
c103740_g1_i2 R	CCTTCGGCTTCACATAATTTTC

Table A3. GluCl and VGSC sequencing primers.

Primer name	Sequence 5'-3'
Nl_GluCl_pri_F	TAATCGCATGCCATGTGTGC
Nl_GluCl_pri_R	GATGTCGCTAGCCGCTTGAT
Nl_GluCl_sec-F	TAGACAGAGCTGAATCCCGC
Nl_GluCl_sec_R	CTTAGTCGCCGACGCTAGTC
Nl_GluCl_seq_F	CTGAGGCCAACAGAGTCTGG
Nl_VGSC_gap_F	TGGGCTTTCCTCTCAGCTTTTC
Nl_VGSC_gap_R	GGAGTTGCGACTAGATCGTG
Nl_VGSC_homologous_repeat_II_F	TCGACATCTTCTGCGTGTGGG
Nl_VGSC_homologous_repeat_II_R	GTAAGATCGAGGTCAGTGTCTC
Nl_VGSC_homologous_repeat_III_F	AATGCTCGGACTGGTGAAGG
Nl_VGSC_homologous_repeat_III_R	GGTCACAGTCGTCCTCGTTT
Nl_VGSC_homologous_repeat_III_seq_F	GCGTCGATACAAATGGCACC
Nl_VGSC_homologous_repeat_III_seq_R	CATTGCGGTCTGGGATGATC

Table A4. *Rdl* sequencing primers.

Primer name	Sequence 5'-3'
Nl_Rdl_generic_1_F	GATGAGGCGCACGTTGGCC
Nl_Rdl_generic_2_F	ATCCAGTTCGTGCGTTCGATG
Nl_Rdl_generic_R	ATCCAGTACATGAGGTTGAAGC
Nl_Rdl_GenBank_R	AGGTCTACTTATCCTCTTCGAG
Nl_Rdl_Alternate_R	GGCCTCCTTACTTATCCGGCT
Nl_Rdl_A301S_R	AGCAACGACGCGAACACCAT
Nl_Rdl_Q359E_F	AGTACGCAACAGTGGGCTAC
Nl_Rdl_Q359E_R	TCCGAAAGCGCTCTACATGA
Dm_Rdl_A301S_F	ATTCAGTTCGTGCGTTCGATG
Dm_Rdl_A301S_R	ACCATAACGAAGCATGTTCCCA
Dm_Rdl_Q359E_F	GTATACGAAACCCACCCGCA
Dm_Rdl_Q359E_R	CACCTCCTGGAACAAGGGTC

Table A5. Generic *CYP6ER1, CYP6AY1* and reference gene qRT-PCR primers.

Primer name	Sequence 5'-3'
Nl_Actin_F	TAACGAGAGGTTCCGTTGCC
Nl_Actin_R	GACAGGACAGTGTTGGCGTA
Nl_α2_tubulin_F	CCACCCTGGAACACTCTGAC
Nl_α2_tubulin_R	CGAAGCAGTGATCGAGGACA
Nl_ER1_generic_F	TCCTGGTGCGCAACTATGAC
Nl_ER1_generic_R	CATCTTGCGGTGCTGATCAC
Nl_AY1_Bass_F	TGCTGAGGCAGAAGATTTCA
Nl_AY1_Bass_R	GACGTCACGCATTTCCAGTA
Nl_AY1_Ding_F	CCAATCACCGCACACCTGGTCAACC
Nl_AY1_Ding_R	GCTTGAGCTGCTATAACACTCTCTG

Table A6. *CYP6ER1* and *CYP4DE1* high fidelity primers and *CYP4DE1* qRT-PCR primers.

Primer name	Sequence 5'-3'
Nl_ER1_Hifi_F	ATGTGGGAAAACTCGTGGTTGGCCTA
Nl_ER1_Hifi_R	AGTGTGAGGTCCTTGTAAGGGTTCAAA
Nl_DE1_BglII_F	TTGGAGATCTATGCGCTTCAAACAGCTATTGG
Nl_DE1_XbaI_R	TACCTCTAGACTAAAAATATGTATCCTCAACAACAGGTG
Nl_DE1_qPCR_F	CACACAGAAGGGTAAAGAACAAG
Nl_DE1_qPCR_R	CATCTGTTTCATCGGACGACA

Table A7. *CYP6ER1* variants qRT-PCR primers

Primer name	Sequence 5'-3'
Nl_ER1_vF_F	CATCCATGAGGTCTACGAAG
Nl_ER1_vF_R	GAGTGCTGAACAGATGGTGT
Nl_ER1_vA_F	CTTTCTTCACCCCCGCCC
Nl_ER1_vA_R	CCTGCATGGTCTCGAACATG
Nl_ER1_vB_F	TCTTGTCACAATCCTGTTGCTG
Nl_ER1_vB_R	TGGATGCATTTCTTGGACAATACG
Nl_ER1_vC_F	GAGACTACTTCTGCATCTTTGT
Nl_ER1_vC_R	GGAAACCATTGGGAAGAATGA
Nl_ER1_vD_F	AGATCAAATCGGCGGATGGA
Nl_ER1_vD_R	CGGAATCATCACTTGAGTTCC
Nl_ER1_vE_F	CCGGAATCATTACTTGAGTTCC
Nl_ER1_vE_R	GTATGATGAGATCAGATCTGTGA

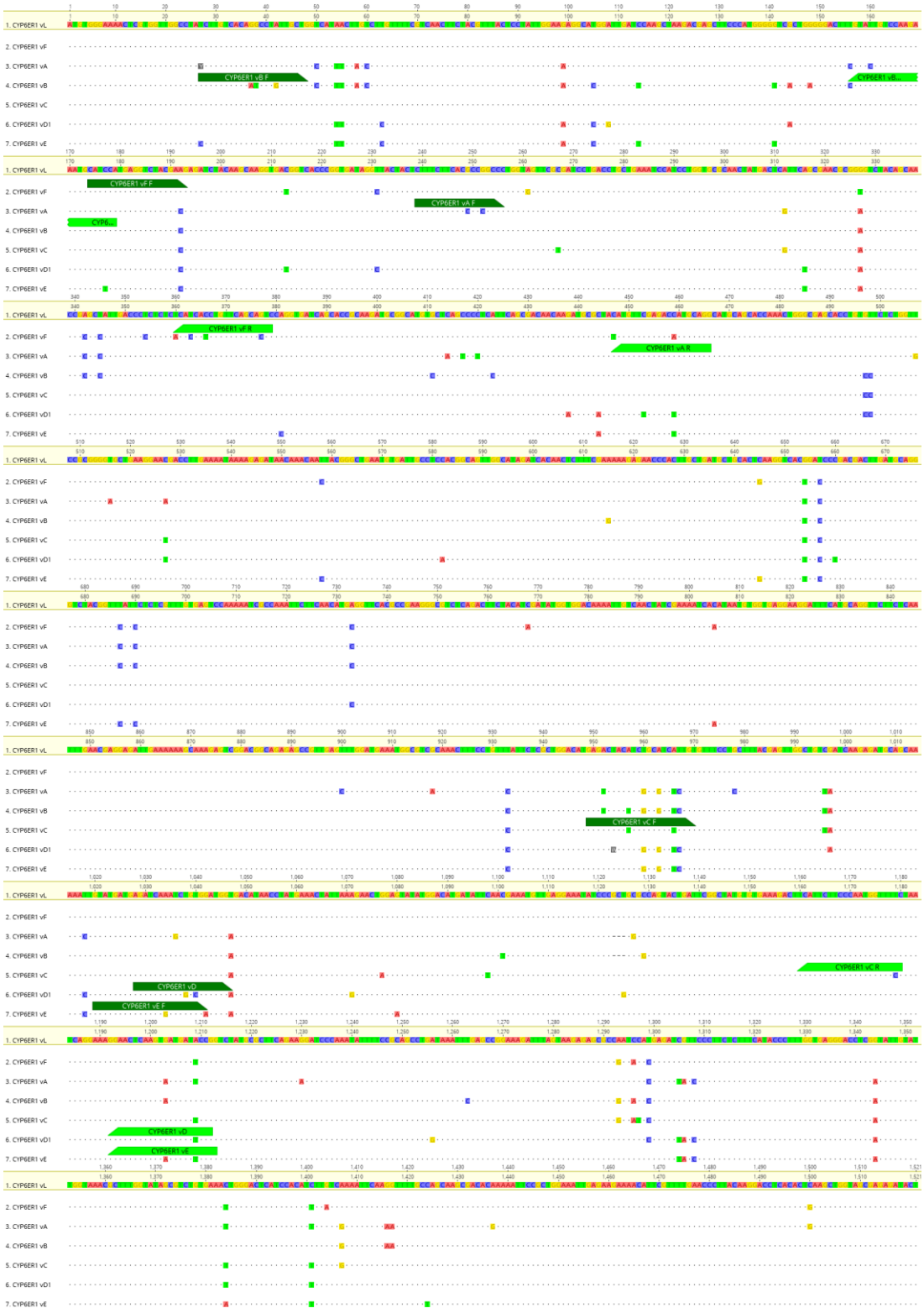

Fig. S1. Nucleotide sequence of the *CYP6ER1* variants and the primer pairs utilised for qRT-PCR

Table A8. Melt peaks (C) for *CYP6ER1* variant specific qRT-PCR.

Gene	Melt peak
Actin	86.5
Tubby	86.7
CYP6ER1vF	87.3
CYP6ER1vA	88.3
CYP6ER1vB	84.5
CYP6ER1vC	82.7
CYP6ER1vD	82.5
CYP6ER1vE	82

Table A9. Top twenty over expressed genes between Bayer-S and Nl33

Bayer-S vs Nl33 Transcript	Blast description
c91225_g1_i1	hypothetical protein TcasGA2_TC030635
c102198_g2_i2	platelet-activating factor acetylhydrolase ib subunit beta-like protein
c90362_g1_i5	single-stranded dna-binding protein 3-like isoform 1
c105136_g2_i1	glucose-6-phosphate isomerase
c100403_g2_i1	regulator of ribosome biosynthesis
c109701_g1_i1	ccr4-not transcription complex subunit 1 isoform x2
c94447_g1_i3	PREDICTED: uricase
c59655_g1_i1	lipase 3-like
c95618_g3_i4	androgen-induced proliferation inhibitor
c91360_g1_i1	adrenodoxin
c92995_g2_i2	fructose-bisphosphate aldolase-like isoform x1
c88625_g1_i1	cullin
c104747_g2_i1	low quality protein: de-cadherin
c101465_g1_i3	v-atpase d
c104523_g1_i7	Putative imidazolonepropionase
c107384_g1_i5	PREDICTED: uncharacterized protein LOC103578838 isoform X1
c100102_g1_i1	trypsin-10
c105545_g1_i4	heterogeneous nuclear ribonucleoprotein l
c110744_g1_i1	pr domain zinc finger protein 1
c91248_g1_i3	cell wall-associated hydrolase

Table A10. Top twenty over expressed genes between Bayer-S and Nl33-eth

Bayer-S vs Nl33-eth	
Transcript	Blast description
c93514_g1_i1	PREDICTED: thioredoxin-2-like
c111588_g2_i3	arylalkylamine n-acetyltransferase
c101021_g2_i1	nadp-dependent malic enzyme
c100139_g1_i1	hypothetical protein HELRODRAFT_186595
c95047_g1_i2	apolipoprotein d
c102636_g1_i1	easter-9
c88625_g1_i1	cullin
c105136_g2_i1	glucose-6-phosphate isomerase
c93223_g1_i1	camp-dependent protein kinase catalytic subunit-like
c97572_g2_i1	PREDICTED: uncharacterized protein LOC101893139
c103516_g1_i4	carboxylesterase, partial
c98716_g1_i1	carboxylesterase-6
c94260_g1_i3	hypothetical protein CERSUDRAFT_40589, partial
c95257_g1_i1	hypothetical protein TcasGA2_TC012174
c107731_g1_i1	PREDICTED: hypothetical protein LOC100744451
c97030_g1_i1	easter-6
c103830_g1_i2	rna recognition motif-containing protein
c92358_g1_i1	hypothetical protein YQE_11485, partial
c99285_g1_i1	serine threonine-protein phosphatase pp1-gamma catalytic subunit-like isoform x1
c104244_g1_i5	3-ketoacyl- mitochondrial

Table A.11. Top twenty over expressed genes between Nl33 and Nl33-eth

Nl33 vs Nl33-eth Transcript	Blast description
c93514_g1_i1	thioredoxin-like protein
c95628_g1_i2	glycine cleavage system h mitochondrial-like
c105273_g1_i2	histone deacetylase
c102776_g3_i1	cell division cycle protein 27 homolog
c103092_g1_i4	lipopolysaccharide-induced tumor necrosis factor-alpha factor homolog
c103756_g1_i2	ras-related protein rab-11a
c99429_g2_i2	serine protease 10
c102396_g1_i2	kelch-like ech-associated protein 1
c99008_g1_i2	PREDICTED: uncharacterized protein LOC101234332
c96181_g2_i8	uncharacterized abhydrolase domain-containing protein ddb_g0269086-like isoform x2
c107868_g1_i3	telomerase-binding protein est1a-like
c111137_g1_i3	oxidoreductase glyr1 homolog
c107699_g1_i2	thyroid receptor-interacting protein 11-like
c99765_g2_i2	retrotransposon protein
c107401_g1_i6	nocturnin isoform x2
c103143_g1_i3	nucleoredoxin-like protein 2
c102684_g2_i13	protein phosphatase 1 regulatory subunit 12a
c95074_g1_i2	transferrin-like isoform x2
c104060_g4_i3	pab-dependent poly -specific ribonuclease subunit 3-like
c110064_g1_i3	oxysterol-binding protein 1 isoform x2

Table A12. Top twenty over expressed genes between Bayer-S and Nl55

Bayer-S vs Nl55 Transcript	Blast description
c83110_g1_i1	polyprotein
c104193_g1_i3	4-coumarate-- ligase 3-like
c101021_g2_i1	nadp-dependent malic enzyme
c89218_g1_i1	rna polymerase
c24277_g1_i1	major core capsid protein
c51844_g1_i1	136.6KD protein
c88625_g1_i1	cullin
c85378_g1_i1	130kd protein
c90362_g1_i5	single-stranded dna-binding protein 3-like isoform 1
c23626_g1_i1	nonstructural protein
c92995_g2_i2	fructose-bisphosphate aldolase-like isoform x1
c105136_g2_i1	glucose-6-phosphate isomerase
c97428_g3_i2	pyrroline-5-carboxylate reductase
c103334_g1_i1	106.4KD protein
c104747_g2_i1	low quality protein: de-cadherin
c100139_g1_i1	hypothetical protein HELRODRAFT_186595
c109685_g2_i1	tricarboxylate transport
c97638_g4_i1	cdgsh iron-sulfur domain-containing protein 2 homolog isoform x1
c93223_g1_i1	camp-dependent protein kinase catalytic subunit-like
c65564_g1_i1	major outer capsid protein

Table A13. Top twenty over expressed genes between Bayer-S and Nl55-eth

Bayer-S vs Nl55-eth	
Transcript	Blast description
c83110_g1_i1	polyprotein
c101021_g2_i1	nadp-dependent malic enzyme
c88625_g1_i1	cullin
c97428_g3_i2	pyrroline-5-carboxylate reductase
c44171_g1_i1	5 -amp-activated protein kinase catalytic subunit alpha-2
c92995_g2_i2	fructose-bisphosphate aldolase-like isoform x1
c100139_g1_i1	hypothetical protein HELRODRAFT_186595
c90362_g1_i5	single-stranded dna-binding protein 3-like isoform 1
c104193_g1_i3	4-coumarate-- ligase 3-like
c93223_g1_i1	camp-dependent protein kinase catalytic subunit-like
c92358_g1_i1	hypothetical protein YQE_11485, partial
c97030_g1_i1	easter-6
c103516_g1_i4	carboxylesterase, partial
c97572_g2_i1	PREDICTED: uncharacterized protein LOC101893139
c95047_g1_i2	apolipoprotein d
c91169_g1_i1	hemolymph protease 2
c107731_g1_i1	PREDICTED: hypothetical protein LOC100744451
c103740_g1_i2	cytochrome partial
c97638_g4_i1	cdgsh iron-sulfur domain-containing protein 2 homolog isoform x1
c104244_g1_i5	3-ketoacyl- mitochondrial

Table A14. Top twenty over expressed genes between Nl55 and Nl55-eth

Nl55 vs Nl55-eth Transcript	Blast description
c109701_g1_i1	ccr4-not transcription complex subunit 1
c82591_g2_i2	ornithine decarboxylase 1-like
c105804_g1_i4	hypothetical protein HELRODRAFT_159370
c105894_g1_i1	pdz domain-containing protein 2
c96852_g1_i2	transcription factor sp4-like
c111720_g1_i2	unconventional myosin-xv isoform x1
c108948_g1_i1	lamin-b receptor
c110971_g1_i5	coiled-coil domain-containing protein cg32809 isoform x10
c109988_g1_i3	gpi transamidase component pig-s
c109363_g3_i3	transient receptor potential cation channel trpm-like
c94005_g4_i12	calcium-dependent secretion activator
c109903_g1_i7	phospholysine phosphohistidine inorganic pyrophosphate phosphatase
c98304_g2_i1	tyrosine-protein phosphatase non-receptor type 14
c105313_g1_i1	dedicator of cytokinesis protein 9
c100622_g1_i1	PREDICTED: uncharacterized protein LOC100680266 isoform X3
c92531_g2_i2	venom protease-like
c107958_g1_i4	fad synthase
c111460_g1_i1	cartilage oligomeric matrix protein
c90079_g1_i1	myb-like protein d
c98169_g2_i2	u4 tri-snrnp-associated protein 1

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Research Article

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Field-evolved resistance to imidacloprid and ethiprole in populations of brown planthopper *Nilaparvata lugens* collected from across South and East Asia

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Abstract

BACKGROUND: We report on the status of imidacloprid and ethiprole resistance in *Nilaparvata lugens* Stål collected from across South and East Asia over the period 2005–2012.

RESULTS: A resistance survey found that field populations had developed up to 220-fold resistance to imidacloprid and 223-fold resistance to ethiprole, and that many of the strains collected showed high levels of resistance to both insecticides. We also found that the cytochrome P450 CYP6ER1 was significantly overexpressed in 12 imidacloprid-resistant populations tested when compared with a laboratory susceptible strain, with fold changes ranging from ten- to 90-fold. In contrast, another cytochrome P450 CYP6AY1, also implicated in imidacloprid resistance, was underexpressed in ten of the populations and only significantly overexpressed (3.5-fold) in a single population from India compared with the same susceptible strain. Further selection of two of the imidacloprid-resistant field strains correlated with an approximate threefold increase in expression of CYP6ER1.

CONCLUSIONS: We conclude that overexpression of CYP6ER1 is associated with field-evolved resistance to imidacloprid in brown planthopper populations in five countries in South and East Asia.

Keywords: brown planthopper; resistance; cytochrome P450; insecticides

1 INTRODUCTION

The brown planthopper (BPH), *Nilaparvata lugens* Stål, is an economically important pest of rice throughout both tropical and temperate zones of South and East Asia. It causes damage to the rice crop via direct phloem-sap feeding, leading to nutrient depletion within the plant, which when infestation levels become high enough manifests as a characteristic stunting, wilting and browning of the affected crop, often referred to as 'hopperburn'. BPH is also an effective vector of a number of rice pathogens, including ragged stunt virus and grassy stunt virus.[1] The resulting cumulative damage to the rice crop can result in a significant (up to 60%) loss of yield in susceptible rice varieties.[2] This is starkly illustrated by the observation that, between 2009 and 2011, rice production in Thailand suffered huge losses due to BPH, with more than 3 million ha infested and in excess of 1.1 million t of paddy, with an export value of an estimated $US 275 million, lost (data published by the International Rice Research Institute).

The control of BPH has for many years predominantly relied on the use of synthetic insecticides. This has resulted in the emergence of populations with high levels of resistance to many of the major classes of insecticides, including the organophosphates, carbamates, pyrethroids, neonicotinoids and phenylpyrazoles.[3–6] Since the early 1990s, the neonicotinoid insecticide imidacloprid has been widely applied throughout Asia for BPH control. Reduced efficacy/resistance to this insecticide emerged in populations across Asia over the period 2003–2006.[7,8] More recent monitoring across nine regions of China showed that imidacloprid resistance levels have again increased, with resistance ratios [LD_{50} field population/LD_{50} susceptible (1995 collected) strain] as high as 617-fold being recorded in 2012.[6] Similar levels of imidacloprid resistance in BPH immigrating into Japan have recently been reported, with resistance ratios of 616-fold (comparing LD_{50} values of populations sampled in 1992 to 2012).[9] Owing to the significant resistance to neonicotinoid insecticides, phenylpyrazole

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(fiprole) insecticides, such as ethiprole and fipronil, which target the gamma-aminobutyric acid (GABA)-gated chloride channel of the insect's central nervous system,[10] have increasingly been used as a substitute for BPH control. However, emerging resistance to fipronil (23.8–43.3-fold resistance) and cross-resistance (47.1–100.9-fold) to ethiprole in field populations of BPH have been reported in China,[11,12] and significant (308.5-fold) levels of resistance to ethiprole in Thailand.[5]

Although the molecular mechanism(s) underlying resistance to fiproles have not been fully characterised,[5] significant progress has been made in characterising the molecular basis of resistance to imidacloprid. Target-site resistance to this compound was described in a laboratory-selected strain of BPH before reports of control failure in the field; however, this mechanism has never been identified in any field-collected population.[13] In contrast, several studies have provided evidence that enhanced cytochrome P450 monooxygenase (P450) activity contributes to the neonicotinoid resistance of field-collected populations of BPH.[14–16] This detoxification mechanism was initially implicated by use of the metabolic enzyme inhibitor piperonyl butoxide (PBO) and the model substrate 7-ethoxycoumarin.[17,15] More recently the overexpression of two candidate P450 enzymes, CYP6ER1 and CYP6AY1, has been linked with imidacloprid resistance.[18,19] In the first study, the expression levels of 32 tentative unique P450s, identified from two recent sequencing projects and by degenerate PCR, were examined in a susceptible *N. lugens* strain and moderately and highly resistant strains from China and Thailand, using quantitative real-time PCR. A single P450 gene, CYP6ER1, was identified as highly overexpressed (up to 40-fold) in all resistant strains compared with the susceptible strain, and the level of expression observed in the different strains was significantly correlated with the resistance phenotype.[18] In the second study, the expression levels of 14 P450 genes were compared between a laboratory strain selected with imidacloprid for 40 generations and a susceptible strain, using quantitative RT-PCR. Six genes were identified as significantly overexpressed in the resistant strain, with CYP6AY1 showing the highest level of overexpression (~18-fold) compared with the susceptible strain.[19] Functional expression of CYP6AY1 and RNAi experiments provided evidence that CYP6AY1 has the capacity to metabolise imidacloprid and confer resistance.[19]

The aim of the present study was to analyse the changing levels of resistance to imidacloprid and ethiprole in *N. lugens* field strains collected from five countries in South and East Asia from 2005 through to 2012, and to investigate the relative roles of CYP6ER1 and CYP6AY1 in the resistance of these strains to imidacloprid.

2 EXPERIMENTAL METHODS

2.1 Insect strains

Baseline susceptibility data were generated using a laboratory-maintained strain of *N. lugens* (Bayer-S) provided by Bayer CropScience (Monheim, Germany). Bayer CropScience also organised the transfer to Rothamsted Research of field strains collected from across South and East Asia between 2005 and 2012. All strains were reared in the laboratory on whole rice plants (*Oryza sativa* L. ssp.) under controlled environmental conditions (26 °C/16 h photoperiod).

2.2 Laboratory selection

Two of the field strains, NL9 and NL39, demonstrating relatively high levels of resistance to imidacloprid, were placed under further selection with imidacloprid in the laboratory. NL9 was reared on rice plants treated with successively higher doses (concentrations ranging between 10 and 180 mg L^{-1}) of imidacloprid over 13 generations, whereas NL39 was placed directly onto rice plants treated with 200 mg L^{-1} imidacloprid and selected over two generations.

2.3 Topical application bioassay (imidacloprid)

Adult macropterous (long-winged) females of *N. lugens* were taken from age-structured populations and were less than 10 days old. Approximately 15 females were lightly anaesthetised and dosed with the required concentration of technical imidacloprid on the upper surface (pronotum) of the prothorax using 0.25 µL of acetone as the solvent carrier, delivered using a hand-held Burkard microapplicator (Burkard Manufacturing Co. Ltd, Rickmansworth, UK) fitted with a 1 cm^3 all-glass syringe. Control insects were dosed with 0.25 µL of acetone only. Treated individuals were placed in 50 mL specimen tubes containing untreated five-week-old rice stems (cut into 10 cm lengths) and contained using a ventilated lid. A small hole (3 mm diameter) was drilled in the base of each of the tubes, which were then stored vertically in a water bath (submerging only the base of each rice stem) in a 16 h photoperiod at 26 °C for 48 h. Insect mortality at 48 h was assessed by eye; adults showing no sign of movement were scored as dead. Bioassays consisted of three replicates at each concentration. Diagnostic doses represented the LD_{95} (4 mg L^{-1}) and 5 × LD_{95} (20 mg L^{-1}) of the susceptible strain.

2.4 Leaf-dip bioassay (ethiprole)

Adult females were taken from age-structured populations and were less than 10 days old. Rice stems (10 cm cut lengths) were dipped into the required concentrations of formulated fiprol insecticide for 20 s, air dried and placed in a plastic specimen tube. Approximately 15 females were aspirated directly into each of the tubes, which were sealed with a ventilated lid. A small hole (3 mm diameter) was drilled in the base of each of the tubes, which were then stored vertically in a water bath (submerging only the base of each stem) in a 16 h photoperiod at 26 °C for 72 h. Mortality was assessed by eye; adults showing no sign of movement were scored as dead. Bioassays consisted of three replicates at each concentration.

2.5 Data analysis

Probit analysis with Genstat 16th Edition software (VSN International Ltd, Hemel Hempstead, UK) was conducted to generate estimated LC_{50} values. Resistance factors were calculated by dividing the LC_{50} of a resistant strain by that of the susceptible strain. Mortality rates at diagnostic concentrations were subjected to Abbott's correction for natural mortality.[20] Standard errors for mortalities at diagnostic concentrations were calculated using a binomial model.

2.6 Real-time quantitative RT PCR

In qRT-PCR analysis of CYP6ER1 and CYP6AY1 expression, primers designed previously[18] and the CYP6AY1 primers employed by Ding *et al.*[19] were used. PCR reactions (15 µL) contained 5 µL of cDNA (2.5 ng), 7.5 µL of SYBR Green JumpStart Taq Readymix (Sigma Aldrich) and 0.25 µM of each primer. Samples were run on a Rotor-Gene 6000 (Corbett Research, Cambridge, UK) using the following temperature cycling conditions: 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s, 57 °C for 15 s and 72 °C for 20 s. A

Appendix B

Table 1. Mortalities (%) (± standard error) for all *Nilaparvata lugens* strains at two diagnostic doses (LD_{95} and $5 \times LD_{95}$ of the susceptible strain) of imidacloprid topically applied to adult females. Highlighted data were previously reported in Gorman *et al.*[7]

				Imidacloprid[a]	
Strain	Year	Country of origin	Region/area	4 mg L^{-1}, 1 ng AI $insect^{-1}$	20 mg L^{-1}, 5 ng AI $insect^{-1}$
Bayer-S	–	–		91.43(±4.48)	100.00 ± nc
CHN-1	2005	China	Nanjing	53.45(±6.39)	100.00 ± nc
IND-1	2005	India	East Godavari District, Andhra Pradesh	85.21(±4.74)	100.00 ± nc
IND-2	2005	India	Karnataka State	91.23(±3.68)	100.00 ± nc
IND-3	2005	India	Mumbai	59.32(±6.09)	100.00 ± nc
IND-4	2005	India	West Godavari District, Andhra Pradesh	83.34(±5.02)	100.00 ± nc
IND-5	2005	India	Bellary District, Karnataka State	59.66(±7.57)	100.00 ± nc
IND-6	2005	India	West Godavari District, Andhra Pradesh	17.98(±4.66)	81.50(±4.74)
IND-7	2005	India	East Godavari District, Andhra Pradesh	18.63(±4.79)	71.40(±5.61)
ISA-1	2005	Indonesia		96.36(±2.50)	100.00 ± nc
MAL-1	2005	Malaysia		54.03(±6.91)	nt
THAI-1	2005	Thailand		87.01(±4.20)	100.00 ± nc
VTN-1	2005	Vietnam		92.10(±3.67)	100.00 ± nc
CHN-2	October 2006	China	Guandong Province	41.41(±7.11)	46.20(±10.40)
CHN-3	October 2006	China	Guangxi Province	23.34(±6.24)	75.81(±6.69)
CHN-4	September 2006	China	Jiangsu Province	55.71(±6.64)	75.11(±6.92)
CHN-5	October 2006	China	Hunan Province	35.00(±6.88)	67.50(±6.76)
IND-8	April 2006	India	Bellary District, Karnataka State	57.53(±8.13)	97.14(±2.36)
IND-9	April 2006	India	East Kolkata, West Bengal	50.00(±7.45)	79.71(±5.15)
IND-10	October 2006	India	West Godavari, Andhra Pradesh	33.67(±6.68)	48.04(±5.97)
IND-11	October 2006	India	East Godavari, Andhra Pradesh	0.00 ± nc	5.75(±4.18)
MAL-2	December 2006	Malaysia	Sabak Bernam District, Selangor	13.87(±6.78)	33.07(±5.23)
THAI-2	August 2006	Thailand	Chainat Province, San Buri District	22.41(±7.74)	35.71(±8.10)
THAI-3	August 2006	Thailand	Suphanburi Province	35.00(±6.88)	67.50(±6.76)
VTN-2	August 2006	Vietnam	Đồng Tháp Province, Tháp Mười District	2.27(±2.52)	0.00 ± nc
VTN-3	August 2006	Vietnam	Long An Province, Bến Lức District	26.63(±7.70)	42.11(±7.81)
NL2	October 2008	India	Bellary District, Karnataka State	100.00 ± nc	83.33(±6.80)
NL3	October 2008	India	Karnataka State	66.67(±8.61)	75.00(±7.91)
NL5	October 2008	Thailand	Samchuk District, Suphanburi Province	86.67(±6.21)	93.33(±4.55)
NL6	October 2008	India	West Medinapuri, West Bengal, East India	51.11(±8.33)	91.75(±4.35)
NL8	December 2008	Vietnam	Tantru District, Long An Province	64.29(±8.75)	82.14(±6.99)
NL9	August 2009	Thailand		26.92(±8.10)	53.85(±9.10)
NL10	September 2009	Indonesia	Subang, West Java	11.90(±5.40)	73.57(±7.45)
NL11	October 2009	India	Sindhanoor, Southern India	6.67(±4.55)	23.33(±7.72)
NL12	October 2009	India	Karnataka State	0.00 ± nc	7.69(±4.87)
NL13	October 2009	India	Nadia District, West Bengal, East India	0.00 ± nc	3.70(±3.45)
NL14	October 2009	India	Hooghly District, West Bengal, East India	0.00 ± nc	68.00(±8.52)
NL15	September 2009	China	Nanning City, Guangxi Province	34.38(±8.67)	82.36(±6.85)
NL16	September 2009	China	Danyang City, Jiangsu Province	2.32 ± nc	3.43 ± nc
NL17	November 2009	China	Wuhan City, Hubei Province	24.24(±7.14)	91.98(±4.66)
NL18	November 2009	China	Fengxin County, Jiangxi Province	39.50(±6.85)	86.15(±6.01)
NL19	December 2009	Indonesia	East Java	0.00 ± nc	25.93(±8.00)
NL20	December 2009	Indonesia	Gabus Pati District, Central Java	10.71(±5.65)	60.71(±8.92)
NL21	March 2010	Thailand	Suphanburi Province, Sriprachan District	16.78(±6.41)	7.05(±3.69)
NL25	October 2010	India	Koppal District, Karnataka State	16.27(±5.26)	37.00(±5.04)
NL27	September 2010	China	Danyang City, Jiangsu Province	66.52(±6.04)	83.26(±4.78)
NL28	September 2010	China	Nanning City, Guanxi Province	68.07(±6.95)	76.48(±6.19)
NL29	October 2010	India	West Bengal	85.51(±5.25)	85.03(±5.14)
NL30	September 2010	China	Nanchang City, Jianxi Province	62.40(±7.96)	79.35(±5.78)
NL31	October 2010	Taiwan	Yulin County	9.94(±4.85)	37.32(±7.75)
NL32	October 2010	China	Foshan City, Guandong Province	75.18(±6.30)	74.64(±6.42)
NL33	November 2010	Vietnam	Trà Vinh Province, Southern Vietnam	2.86(±2.64)	21.80(±6.70)

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Table 1. Continued

Strain	Year	Country of origin	Region/area	Imidacloprid[a] 4 mg L^{-1}, 1 ng AI $insect^{-1}$	Imidacloprid[a] 20 mg L^{-1}, 5 ng AI $insect^{-1}$
NL34	April 2011	India	Koppal District, Karnataka State	61.38(±6.34)	85.96(±4.52)
NL35	April 2011	India	Koppal District, Karnataka State	100.00 ± nc	96.50(±2.56)
NL39	August 2011	Vietnam	Hau Giang	2.00 ± nc	1.00 ± nc
NL40	August 2011	Indonesia	Anjatan District, Indramayu	8.90(±4.96)	27.32(±7.53)
NL41	August 2011	Indonesia	Binong District, Subang	23.75(±6.42)	45.67(±8.08)
NL42	August 2011	Indonesia	Gegesik District, Cirebon	26.67(±6.99)	41.09(±7.50)
NL43	August 2011	Indonesia	Binong District, Subang	19.44(±5.90)	46.02(±7.51)
NL44	August 2011	Indonesia	Parnanukan District, Subang	2.48(±2.52)	4.45(±3.22)
NL45	September 2011	India	Raipur, Chhattisgarth	30.14(±8.11)	34.25(±8.14)
NL46	October 2011	India	Mohanpur, West Bengal	10.00(±5.30)	28.00(±8.20)
NL47	September 2011	China	Xi Jiao District, Danyang City, Jiangsu Province	18.92(±6.44)	50.00(±8.45)
NL52	March 2012	India	Koppal District, Karnataka State	15.13(±5.67)	58.31(±8.00)
NL53	March 2012	India	West Godavari District, Andhra Pradesh	45.88(±7.88)	67.00(±7.34)
NL54	March 2012	India	Karimnagar, Warangar District	36.83(±7.58)	78.06(±6.63)
NL55	April 2012	India	East Godavari District, Andhra Pradesh	40.00(±7.95)	60.00(±7.95)
NL56	April 2012	India	East Godavari District, Andhra pradesh	62.11(±7.77)	67.33(±7.24)
NL57	October 2012	India	Kanagala District, Karnataka State	24.51(±7.38)	31.06(±6.98)
NL58	October 2012	India	Mudhapur, Karnataka State	29.41(±7.03)	32.86(±6.93)
NL59	October 2012	India	Sidhikerra, Karnataka State	15.74(±5.69)	42.42(±8.02)

[a] nt = not tested; nc = not calculable.

final melt-curve step was included post-PCR (ramping from 72 to 95 °C by 1 °C every 5 s) to check for non-specific amplification. Each qRT-PCR experiment consisted of three independent biological replicates, with two technical replicates for each. Technical replication was limited to two replicates, (1) as PCR reactions were set up using a liquid handling robot (CAS 1200; Corbett Research) which provided high levels of technical reproducibility, and (2) to allow us to employ a sample maximisation strategy (i.e. running as many samples as possible in the same run in order to minimise technical run-to-run variation). Data were analysed according to the $\Delta\Delta C_t$ method.[21] For normalisation, two reference genes were validated experimentally for each strain, actin and α2-tubulin, with the geometric mean of the selected genes then used for normalisation according to the strategy described previously.[22]

3 RESULTS AND DISCUSSION

3.1 Development of imidacloprid resistance in *N. lugens* populations from 2005 to 2012

As previously reported,[7] responses of 2005 field-collected samples of *N. lugens* to imidacloprid showed variation, particularly at the lower (4 mg L^{-1}) dose tested (Table 1), with some strains appearing susceptible but other strains showing the first indications of a resistance problem. Strains collected that exhibited a decreased susceptibility to imidacloprid at the higher (20 mg L^{-1}) diagnostic concentration (IND-6 and IND-7) were analysed for the presence of the Y151S mutation, known to reduce the agonist potency of a range of neonicotinoid insecticides, including imidacloprid.[13] Using PCR-based techniques, it was shown that, at the Y151S mutation site, individuals of both strains expressed 'wild-type' base pairings, i.e. there was no evidence for Y151S-mediated target-site resistance as recently described for a laboratory-selected strain.

Table 2. Dose–response data for *Nilaparvata lugens* laboratory susceptible (Bayer-S) and imidacloprid-resistant strains against imidacloprid topically applied to adult females

Strain	Year	Country	Imidacloprid LD_{50} (95% limits) (ng AI $insect^{-1}$)	Imidacloprid RR[a]
Bayer-S			0.61 (0.46–0.79)	1.0
CHIN-1	2005	China	6.06 (4.82–7.55)	10.0
IND-3	2005	India	4.47 (3.41–5.71)	7.4
IND-5	2005	India	7.20 (6.60–7.83)	11.9
IND-6	2005	India	11.09 (9.62–12.78)	18.3
IND-7	2005	India	13.65 (11.42–16.07)	22.5
MAL-1	2005	Malaysia	3.46 (3.04–3.92)	5.7
IND-11	2006	India	58.68 (31.83–97.77)	96.7
NL2	2008	India	0.80 (0.15–2.35)	1.3
NL3	2008	India	0.86 (0.06–3.53)	1.4
NL6	2008	India	24.10 (1.15–259.09)	39.7
NL8	2008	Vietnam	2.52 (0.17–9.77)	4.2
NL9	2009	Thailand	97.00 (3.40–434.00)	139.0
NL11	2009	India	10.98 (2.18–31.00)	18.0
NL15	2009	China	20.12 (1.14–243.60)	33.1
NL16	2009	China	29.80 (5.98–64.50)	49.1
NL25	2010	India	38.88 (1.06–323.60)	64.1
NL27	2010	China	42.41 (15.61–87.62)	69.1
NL30	2010	China	133.80 (59.9–277.00)	220.4
NL32	2010	China	60.59 (31.25–85.59)	99.8

[a] RR = resistance ratio (R/S).

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Appendix B

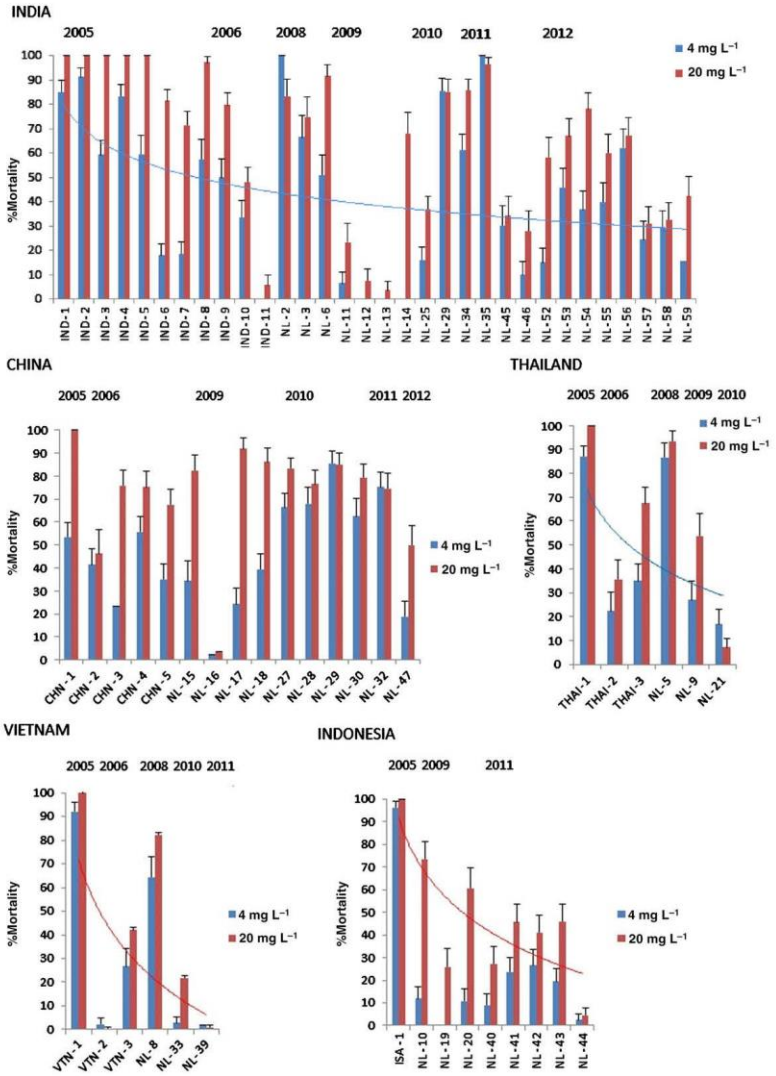

Figure 1. Mortalities (%) (± standard error) at two discriminating doses of imidacloprid for field-collected strains of *N. lugens*.

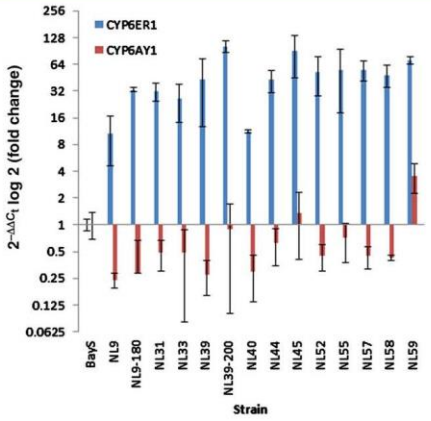

Figure 2. Fold change in expression of CYP6ER1 and CYP6AY1 in 14 resistant *N. lugens* strains compared with the susceptible reference Bayer-S as determined by quantitative real-time PCR. Error bars display 95% confidence intervals.

In contrast to 2005, all 13 field samples collected in 2006 showed reduced susceptibility to imidacloprid at both diagnostic doses. Responses at 4 mg L^{-1} ranged from 0 to 60% mortality, and those at 20 mg L^{-1} from 0 to 97% mortality. The most resistant samples, IND-11, VTN-2, MAL-2, THAI-2 and CHN-2, originated from different countries (India, Vietnam, Malaysia, Thailand and China), leading to the conclusion that resistance was neither confined to nor focused within a specific geographical region. This widespread distribution is, however, consistent with the migratory behaviour of *N. lugens*. To assess the potency of the mechanism(s) responsible, dose–response data for one of the most resistant samples (IND-11) were generated. A comparison between the laboratory susceptible strain (S) and strain IND-11 showed near-parallel response lines,[7] with a resistance ratio of 96.7 at LD_{50}. Approximately 30% of the IND-11 individuals were capable of surviving 100 mg L^{-1} (25 ng AI $insect^{-1}$), which relates to 25 × LD_{95} of the susceptible strain. As in 2005, results for two of the most imidacloprid-resistant strains collected in 2006 (CHN-2 and THAI-2) also disclosed 'wild-type' sequences at the Y151S mutation site.

A limited number of field samples collected in 2008 from India, Thailand and Vietnam suggested that resistance was not as high in individual strains as in 2006. However, for field samples collected in 2009, responses at 4 mg L^{-1} ranged from 0 to 40% mortality, and those at 20 mg L^{-1} from 4 to 92% mortality. Again, highly resistant samples were identified as originating from different countries (India, China, Indonesia and Thailand), suggesting that the resistance problem across South and East Asia had not really abated. LD_{50} analysis of strain NL9 from Thailand indicated a resistance ratio of 139 (Table 2), roughly comparable with that reported for IND-11 in 2006. Resistance to imidacloprid appeared to stabilise in 2010, but a highly resistant sample NL30, with an LD_{50} resistance ratio of 220, was collected from China, indicating that in some strains the potency of resistance to imidacloprid was continuing to increase. Since 2010, resistance to imidacloprid has continued to persist in field-collected strains (Table 1), and is clearly entrenched in BPH populations.

Analysis of imidacloprid resistance development in the individual countries of India, Thailand, Indonesia and Vietnam, based on the responses of collected field strains to discriminating doses of imidacloprid, indicates a clear trend towards high resistance (Fig. 1). For China, however, the trend is less clear. This may be because BPH cannot overwinter in subtropical and temperate regions north of 22° N, and immigrate into China from other regions during the autumn months.

3.2 Association of overexpression of CYP6ER1 and CYP6AY1 with resistance to imidacloprid

As detailed in the introduction, two cytochrome P450s have previously been linked with imidacloprid resistance in a small number of BPH laboratory and field populations. In the present study the expression levels of these two P450s were explored in 12 field populations collected from a range of countries in Asia (from 2009 to 2012) that exhibited clear resistance to imidacloprid in discriminating dose bioassays (Fig. 1, Table 1). As shown in Fig. 2, CYP6ER1 was significantly overexpressed in all 12 resistant populations when compared with a lab susceptible strain, with fold changes ranging from ten- to 90-fold. In contrast, CYP6AY1 was underexpressed in ten of the populations compared with the same susceptible strain, and was only significantly overexpressed (3.5-fold) in a single population from India (NL59). To see whether selection of the field strains with imidacloprid caused any increase in the expression levels of CYP6ER1 or CYP6AY1, two field strains (NL9 and NL39) were selected with imidacloprid up to final concentrations of 180 and 200 mg L^{-1} imidacloprid respectively. When the expression levels of CYP6ER1 were compared between NL9 (unselected) and NL9-180 (selected), the expression level was found to have significantly increased threefold after selection, rising from ~11- to 33-fold. A similar effect was seen for NL39 (unselected) versus NL39-200 (selected), with an increase from 43- to 103-fold overexpression. Also noteworthy is that variation in the level of expression of CYP6ER1 among individual biological replicates decreased considerably after selection (as indicated by significantly reduced 95% confidence limits – see Fig. 2), suggesting that selection has reduced genetic heterogeneity in this strain and that all replicates overexpress this CYP at a universally high level. After selection, CYP6AY1 expression increased (see Fig. 2) from 0.24 in NL9 to 0.29 in NL9-180 and from 0.28 in NL39 to 0.91 in NL39-200; however, the difference in expression between both unselected/selected strains was not statistically significant as a result of significant variation in the expression levels of this CYP observed between biological replicates, particularly in the case of NL39-200, and expression levels remained below that of the susceptible strain.

These results provide further evidence that overexpression of CYP6ER1 contributes to imidacloprid resistance in BPH throughout

Table 3. Fold change in expression of CYP6AY1 in five imidacloprid-resistant *N. lugens* strains compared with the susceptible reference Bayer-S as determined by quantitative real-time PCR

Strain	Fold change ($2^{-\Delta\Delta C_T}$)	95% confidence level
Bayer-S	1.06	0.45
NL9	0.30	0.10
NL9-180	0.20	0.28
NL39	0.25	0.06
NL39-200	0.50	0.34
NL59	0.78	0.35

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Appendix B

Table 4. Mortalities (%) (± standard error) for all *Nilaparvata lugens* strains at two diagnostic doses (LC_{95} and 5 × LC_{95} of the susceptible strain) of ethiprole by leaf-dip bioassay

Strain	Year	Country of origin	Region/area	Ethiprole[a]	
				3 mg L^{-1}	15 mg L^{-1}
Bayer-S	–	–		100.00 ± nc	100.00 ± nc
CHN-1	2005	China	Nanjing	100.00 ± nc	nt
IND-1	2005	India	East Godavari District, Andhra Pradesh	98.04(±1.90)	100.00 ± nc
IND-2	2005	India	Karnataka State	100.00 ± nc	100.00 ± nc
IND-3	2005	India	Mumbai	96.55(±2.37)	100.00 ± nc
IND-4	2005	India	West Godavari District, Andhra Pradesh	96.23(±2.59)	100.00 ± nc
IND-5	2005	India	Bellary District, Karnataka State	100.00 ± nc	nt
IND-6	2005	India	West Godavari District, Andhra Pradesh	94.90(±4.58)	100.00 ± nc
IND-7	2005	India	East Godavari District, Andhra Pradesh	85.08(±6.23)	100.00 ± nc
ISA-1	2005	Indonesia		98.14(±1.82)	100.00 ± nc
MAL-1	2005	Malaysia		95.00(±4.95)	nt
THAI-1	2005	Thailand		89.98(±4.13)	100.00 ± nc
VTN-1	2005	Vietnam		100.00 ± nc	100.00 ± nc
IND-11	October 2006	India	East Godavari, Andhra Pradesh	33.59(±8.35)	nt
NL2	October 2008	India	Bellary District, Karnataka State	92.00(±4.95)	nt
NL3	October 2008	India	Karnataka State	80.00(±7.30)	nt
NL5	October 2008	Thailand	Samchuk District, Suphanburi Province	0.00 ± nc	nt
NL6	October 2008	India	West Medinapuri, West Bengal, East India	83.33(±6.80)	nt
NL8	December 2006	Vietnam	Tantru District, Long An Province	26.67(±8.07)	nt
NL9	August 2009	Thailand		41.67(±9.00)	nt
NL10	September 2009	Indonesia	Subang, West Java	24.35(±7.84)	nt
NL11	October 2009	India	Sindhanoor, Southern India	77.26(±6.39)	nt
NL12	October 2009	India	Karnataka State	42.86(±8.89)	nt
NL13	October 2009	India	Nadia District, West Bengal, East India	96.72(±2.97)	nt
NL14	October 2009	India	Hooghly District, West Bengal, East India	76.13(±8.20)	nt
NL15	September 2009	China	Nanning City, Guangxi Province	0.00 ± nc	nt
NL16	September 2009	China	Danyang City, Jiangsu Province	7.41(±4.78)	nt
NL17	November 2009	China	Wuhan City, Hubei Province	14.81(±6.49)	nt
NL18	November 2009	China	Fengxin County, Jiangxi Province	7.41(±4.78)	nt
NL19	December 2009	Indonesia	East Java	23.08(±7.69)	nt
NL20	December 2009	Indonesia	Gabus Pati District, Central Java	48.53(±9.28)	nt
NL21	March 2010	Thailand	Suphanburi Province, Sriprachan District	24.29(±7.07)	nt
NL25	October 2010	India	Koppal District, Karnataka State	49.87(±7.07)	nt
NL27	September 2010	China	Danyang City, Jiangsu Province	39.20(±7.28)	nt
NL28	September 2010	China	Nanning City, Guangxi Province	42.95(±7.38)	nt
NL29	October 2010	India	West Bengal	100.00 ± nc	nt
NL30	September 2010	China	Nanchang City, Jiangxi Province	36.30(±7.17)	nt
NL32	October 2010	China	Foshan City, Guandong Province	33.58(±6.96)	nt
NL33	November 2010	Vietnam	Trà Vinh Province, Southern Vietnam	6.72(±3.82)	nt
NL34	April 2011	India	Koppal District, Karnataka State	58.35(±6.65)	nt
NL35	April 2011	India	Koppal District, Karnataka State	90.71(±4.38)	nt
NL39	August 2011	Vietnam	Hau Giang	3.64(±3.12)	0.00 ± nc
NL40	August 2011	Indonesia	Anjatan District, Indramayu	8.59(±4.55)	5.82(±3.80)
NL41	August 2011	Indonesia	Binong District, Subang	34.80(±7.94)	39.82(±7.84)
NL42	August 2011	Indonesia	Gegesik District, Cirebon	25.93(±7.30)	24.32(±7.05)
NL43	August 2011	Indonesia	Binong District, Subang	11.42(±5.30)	38.44(±8.00)
NL44	August 2011	Indonesia	Parnanukan District, Subang	34.15(±7.32)	46.12(±7.69)
NL45	September 2011	India	Raipur, Chhattisgarth	68.66(±7.43)	89.14(±4.64)
NL46	October 2011	India	Mohanpur, West Bengal	56.87(±7.47)	82.64(±5.65)
NL47	September 2011	China	Xi Jiao District, Danyang City, Jiangsu City	15.15(±5.41)	21.92(±6.17)
NL52	March 2012	India	Koppal District, Karnataka State	12.50(±5.51)	50.00(±8.33)
NL53	March 2012	India	West Godavari District, Andhra Pradesh	74.13(±7.01)	89.06(±4.88)
NL54	March 2012	India	Karimnagar, Warangal District	75.85(±6.53)	81.22(±5.96)
NL55	April 2012	India	East Godavari District, Andhra Pradesh	13.89(±5.69)	8.64(±4.68)
NL56	April 2012	India	East Godavari District, Andhra Pradesh	36.11(±7.69)	30.79(±7.69)
NL57	October 2012	India	Kanagala Camp, Karnataka State	30.00(±7.07)	65.00(±7.36)
NL58	October 2012	India	Mudhapur, Karnataka State	45.95(±8.19)	55.26(±8.07)
NL59	October 2012	India	Sidhikerra, Karnataka State	35.82(±7.78)	63.33(±7.82)

[a] RR = resistance ratio (R/S).

Table 5. Dose–response data for *Nilaparvata lugens* laboratory susceptible (S) and fiprol-resistant strains against ethiprole applied as a leaf dip to adult females

			Ethiprole		Fipronil	
Strain	Year	Country	LC_{50} (95% limits)	RR[a]	LC_{50} (95% limits)	RR
Bayer-S			0.41 (0.29–0.54)	1	1.16 (0.70–1.66)	1
NL3	2008	India	0.74 (0.52–1.06)	1.8	1.61 (1.27–2.03)	1.4
NL5	2008	Thailand	27.35 (10.56–55.50)	66.8	33.12 (9.70–76.46)	28.5
NL6	2008	India	0.21 (0.12–0.35)	0.51	1.48 (0.15–6.30)	1.3
NL8	2008	Vietnam	41.01 (13.05–116.75)	100	24.33 (6.28–79.06)	20.9
NL9	2009	Thailand	25.56 (5.23–62.57)	62.8	14.49 (7.34–27.56)	12.5
NL10	2009	Indonesia	8.06 (3.38–17.73)	19.8	50.17 (16.52–125.30)	43.3
NL11	2009	India	0.30 (0.002–1.85)	0.72	4.28 (1.79–7.79)	3.7
NL12	2009	India	21.01 (7.67–49.19)	51.6	1.45 (0.87–2.18)	1.3
NL13	2009	India	0.06 (0.00–0.26)	0.15	0.25 (0.16–0.35)	0.2
NL14	2009	India	1.06 (0.30–3.09)	2.6	2.61 (0.73–4.77)	2.3
NL15	2009	China	56.30 (29.10–108.20)	138.3	70.07 (2.35–356.30)	60.5
NL16	2009	China	90.73 (20.55–205.50)	222.9	78.41 (18.71–203.60)	67.7
NL17	2009	China	74.23 (33.43–132.80)	182.4	16.37 (14.20–18.34)	14.1
NL18	2009	China	33.06 (5.974–222.77)	81.2	16.61 (12.94–19.43)	14.3
NL19	2009	Indonesia	33.66 (3.62–105.50)	82.70	6.92 (1.27–21.85)	6.0
NL20	2009	Indonesia	42.10 (2.59–142.10)	103.4	47.71 (11.93–122.40)	41.2
NL21	2009	Thailand	13.02 (5.76–21.95)	32.0	8.21 (1.61–22.94)	7.1

[a] RR = resistance ratio (R/S).

South and East Asia. The results for CYP6AY1 were surprising, and so to confirm this finding we ordered the primer pair used previously to measure CYP6AY1 expression in the study by Ding *et al.*[19] and repeated the qPCR experiments on the NL9, NL9-180, NL39, NL39-200 and NL59 strains. The results of this experiment confirmed our initial findings, with CYP6AY1 downregulated in all strains compared with the susceptible strain, including NL9-180 and NL39-200, the two selected strains (see Table 3). The previous study reporting this P450 as overexpressed used a resistant strain, originally collected from a field population in China that had been continuously selected in the laboratory with imidacloprid over 40 generations. Expression of CYP6AY1 in this strain was compared with a lab susceptible strain, and no comparison was made with the 'unselected' parental line of the resistant strain. However, screening of four field populations from China also showed that CYP6AY1 was significantly overexpressed (4–9-fold). It is possible that CYP6AY1 is overexpressed in *N. lugens* populations in China and not the rest of Asia. In our study, all resistant field strains were compared with a single reference lab susceptible strain, as it is now very difficult to obtain BPH field strains that are susceptible to imidacloprid. Further investigation of the relative roles of CYP6ER1 and CYP6AY1 in imidacloprid resistance by comparing resistant strains with additional susceptible laboratory strains, or field strains if they can be sourced, is required to confirm our findings. Finally, although the results of the present study provide further evidence of a role for CYP6ER1 in imidacloprid resistance, functional characterisation of this P450 to confirm its ability to detoxify imidacloprid is now required.

3.3 Development of ethiprole resistance in *N. lugens* populations from 2005 to 2012

There was no significant variation in the responses of field samples collected in 2005 to the diagnostic concentrations of ethiprole (Table 4). Mortality of all strains was over 85% at 3 mg L^{-1} (LC_{95} of the susceptible strain) and 100% at 15 mg L^{-1} ($5 \times LC_{95}$ of the susceptible strain). In 2006, a field sample from India (IND-11) displaying high levels of resistance to imidacloprid also survived a 3 mg L^{-1} discriminating dose bioassay with ethiprole (34% mortality), indicating an emerging resistance problem. This was confirmed in 2008, when field samples NL5 and NL8 from Thailand and Vietnam had a significant number of survivors (0 and 27% mortality respectively) when bioassayed with 3 mg L^{-1} of ethiprole. A full dose–response analysis of these two strains indicated LC_{50}-based resistance ratios for ethiprole of 67- and 100-fold respectively, and 28.5- and 21-fold respectively for fipronil (Table 5). In 2009, all four field samples collected from China had significant ethiprole resistance (0–14% mortality at a discriminating dose of 3 mg L^{-1}), with LC_{50} resistance ratios for ethiprole ranging from 81- to 223-fold and the corresponding resistance ratios for fipronil ranging from 14- to 68-fold (Table 4).

For the 2010 and 2011 seasons, some apparently susceptible populations (NL29, NL35) were collected from India, but the general trend across South and East Asia indicated a developing resistance problem. Sample NL39, collected in 2011 from Vietnam (and having high levels of imidacloprid resistance), had 0% mortality at a higher (15 mg L^{-1}) discriminating dose of ethiprole. Similarly, sample NL40, collected from Indonesia, also displayed good survivability (6% mortality) at the higher discriminating dose.

As for imidacloprid resistance, analysis of ethiprole resistance development in the individual countries of India, Thailand, Indonesia and Vietnam, based on the responses of collected field strains to discriminating doses of ethiprole (Fig. 3), indicates a clear trend towards high resistance. For China, however, the trend is again less clear, but ethiprole resistance is undoubtedly a major problem in this country.

The molecular mechanisms underlying resistance to ethiprole have not been characterised; however, work on a resistant strain from Thailand suggested that enhanced expression of P450s and

Appendix B

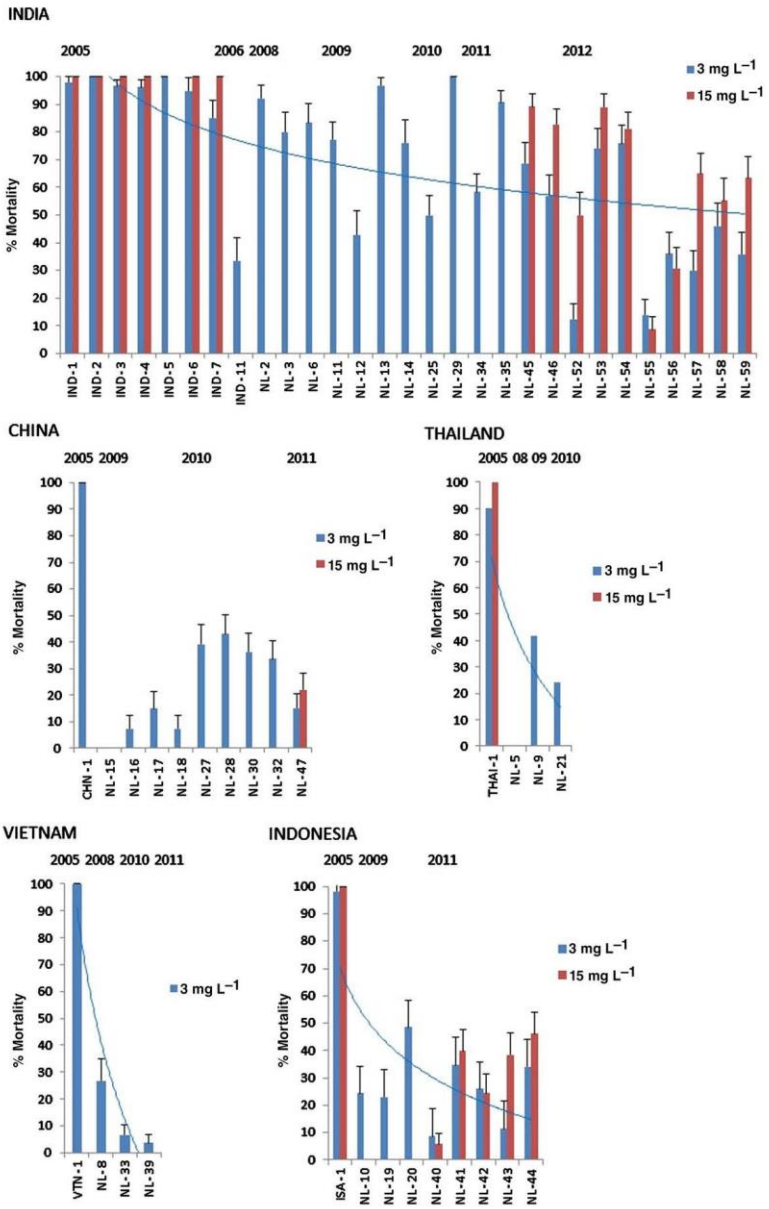

Figure 3. Mortalities (%) (± standard error) at two discriminating doses of ethiprole for field-collected strains of *N. lugens*.

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esterases may contribute to resistance.[5] Although many of the samples analysed in the present study were highly resistant to imidacloprid, there is no evidence to date for a cross-resistance problem involving CYP6ER1.

4 CONCLUSIONS

At present there is no evidence of a common cross-resistance resistance between these two chemical classes of insecticide; however, there is evidence that individual planthoppers may exhibit multiple mechanisms of resistance to the different insecticide modes of action. Our results reveal that overexpression of the cytochrome P450 CYP6ER1 is associated with imidacloprid resistance in BPH populations in five countries in South and East Asia.

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Influence of the RDL A301S mutation in the brown planthopper *Nilaparvata lugens* on the activity of phenylpyrazole insecticides

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ABSTRACT

We discovered the A301S mutation in the RDL GABA-gated chloride channel of fiprole resistant rice brown planthopper, *Nilaparvata lugens* populations by DNA sequencing and SNP calling via RNASeq. Ethiprole selection of two field *N. lugens* populations resulted in strong resistance to both ethiprole and fipronil and resulted in fixation of the A301S mutation, as well as the emergence of another mutation, Q359E in one of the selected strains. To analyse the roles of these mutations in resistance to phenylpyrazoles, three *Rdl* constructs: wild type, A301S and A301S + Q359E were expressed in *Xenopus laevis* oocytes and assessed for their sensitivity to ethiprole and fipronil using two-electrode voltage-clamp electrophysiology. Neither of the mutant *Rdl* subtypes significantly reduced the antagonistic action of fipronil, however there was a significant reduction in response to ethiprole in the two mutated subtypes compared with the wild type. Bioassays with a *Drosophila melanogaster* strain carrying the A301S mutation showed strong resistance to ethiprole but not fipronil compared to a strain without this mutation, thus further supporting a causal role for the A301S mutation in resistance to ethiprole. Homology modelling of the *N. lugens* RDL channel did not suggest implications of Q359E for fiprole binding in contrast to A301S located in transmembrane domain M2 forming the channel pore. Synergist bioassays provided no evidence of a role for cytochrome P450s in *N. lugens* resistance to fipronil and the molecular basis of resistance to this compound remains unknown. In summary this study provides strong evidence that target-site resistance underlies widespread ethiprole resistance in *N. lugens* populations.

1. Introduction

The brown planthopper (BPH), *Nilaparvata lugens* Stål (Hemiptera: Delphacidae), is a key economic pest of rice (*Oryza sativa* L.) throughout Asia. It is a monophagous herbivore and affects the rice crop through direct feeding causing nutrient depletion in the plant. This causes a series of deleterious effects that leads to 'hopperburn', which is characterised by visible stunting, wilting and browning of the affected crop. BPH is also an efficient vector for various rice viruses, including ragged rice stunt and grassy stunt virus [1]. These combined can cause significant damage to rice crops, with up to 60% loss of yield in susceptible cultivars [2].

The application of chemical insecticides has been the preferred method to control BPH, however, this has inevitably led to the evolution of resistance and a reduction in effectiveness. Resistance has affected many of the major classes of insecticides including organophosphates, carbamates, pyrethroids, neonicotinoids and phenylpyrazoles [3–6]. Understanding the levels of resistance through monitoring and analysing the mechanisms responsible for this resistance is a core concept behind being able to effectively control BPH through resistance management strategies.

The phenylpyrazole (fiprole) insecticides, such as ethiprole and fipronil were introduced for BPH control after resistance to imidacloprid became commonplace [7]. Phenylpyrazoles are described as non-competitive blockers of the *gamma*-aminobutyric acid (GABA)-gated chloride channel, a member of the pentameric transmembrane cys-loop ligand-gated ion channel family mediating synapse inhibition in the insect central nervous system [8–10]. Fiproles are potent inhibitors of GABA-mediated inhibitory nerve transmission and belong to group 2 of the MoA classification scheme of the Insecticide Resistance Action Committee (IRAC), that encompasses GABA-gated chloride channel antagonists [11]. This MoA class also includes much older insecticide chemistry, such as the cyclodiene hydrochlorines, which include

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endosulfan and dieldrin [12]. Ethiprole is structurally similar to fipronil only differing in an ethylsulfinyl substituent replacing the trifluoromethylsulfinyl moiety in fipronil [13].

Structural change by replacements of alanine 301 in the GABA-gated chloride channel, encoded for by the *Rdl* (*Resistance to dieldrin*) gene, has been linked to high levels of resistance to insecticidal antagonists, in particular cyclodiene organochlorines [14]. The most common substitution at this position, A301S, was first identified in *Drosophila melanogaster* and shown to cause 4000-fold resistance to dieldrin [15]. However, the role of this mutation in resistance to the newer fiprole insecticides has been debated [16,17]. Other mutations at this amino acid residue, situated in the M2 transmembrane domain, have also been associated with fipronil resistance. A 20,000-fold fipronil resistant strain of *Drosophila simulans* exhibited a A301G replacement at this position in combination with a substitution at a second site, T350M in the M3 domain [18]. Functional expression of *Rdl* in *Xenopus* oocytes showed that the A301G mutation has modest effects on fipronil action, while a receptor variant with both of the mutations exhibited higher levels of resistance to fipronil [18]. A third substitution at the A301 position, A301N (A2'N), has been recently associated with fipronil resistance in two other rice planthopper species, *Sogatella furcifera* and *Laodelphax striatellus* [19,20]. In the former species the A301N mutation was identified in association with a R340Q mutation in the cytoplasmic loop between M3 and M4 of the *S. furcifera* RDL with membrane potential assays suggesting the influence of the double mutation on fipronil resistance was more profound than that of the A301N alone [21]. This finding parallels that of the earlier work in *Drosophila* suggesting two mutations in RDL, one at AA residue 301 and one elsewhere act in concert to influence the level of in vivo resistance to fipronil [16]. However, in contrast to these findings other electrophysiological in vitro studies have revealed no significant differences in fipronil antagonist potency between wildtype and A301S RDL variants expressed in *Xenopus* oocytes [22,23].

Very recently the A301S mutation was also identified in *N. lugens* and correlated with low levels of resistance to fipronil (5-fold in the presence of enzyme inhibitors and 23-fold without) [24]. The authors of this study also identified a second substitution in TM2 (R299Q) that in combination with A301S, was associated with much higher levels of resistance in a laboratory selected strain (96-fold with synergists, 237-fold without). Expression of recombinant RDL receptors, showed the R299Q mutation has a profound effect on the normal functioning of the receptor in response to the endogenous agonist GABA, suggestive of a strong fitness cost. However, the deleterious effects of R299Q was reduced in the presence of the A301S mutation. Surprisingly, the R299Q substitution was identified at extremely low frequency in field populations of *N. lugens* suggesting this is not the main mechanism of resistance in field populations [24].

Due to the evolution of resistance to fipronil in populations of *N. lugens* throughout Asia, and potential issues with the environmental toxicity of this insecticide, most growers subsequently switched to using ethiprole [25,26]. Unfortunately, the rapid uptake of this insecticide has led to recent reports of resistance [5]. To date, the molecular basis of resistance to this insecticide has not been characterised and the potential role of mutations in the GABA-receptor remain unexplored. Metabolic resistance has been implicated in an ethiprole resistant BPH field strain from Thailand [27], though the authors also speculated that GABA receptor mutations could play a role in ethiprole resistance. Another study implicated two cytochrome P450s, CYP4DE1 and CYP6CW3v2, in ethiprole resistance in L. *striatellus* [28].

The aim of this study was to screen the *Rdl* gene for potential mutations in phenylpyrazole resistant BPH field and laboratory selected strains. We report here on the identification of the A301S mutation and a novel mutation, Q359E, and examine their role in fiprole resistance in vivo and in vitro. The potency of these mutations in causing ethiprole resistance was further assessed in *D. melanogaster*.

2. Material and methods

2.1. N. lugens strains and laboratory selection

The laboratory maintained strain of *N. lugens* (Bayer-S) was provided by Bayer CropScience (Monheim, Germany). The field strains NI33 (South Vietnam, collected November 2010) and NI55 (East Godavari District, Andhra Pradesh, India, collected February 2012) were provided by Bayer CropScience. NI33 and NI55 demonstrated high levels of resistance to ethiprole and were then placed under further selection with ethiprole in the laboratory. Strains of NI33 and NI55 were reared on rice plants sprayed with successively higher concentrations (ranging between 7.5 and 100 mg L^{-1}) of ethiprole over 15 generations. A second culture of NI33 and NI55 was maintained on untreated rice plants. All strains were reared in the laboratory on whole rice plants (*O. sativa* L. ssp.) under controlled environmental conditions (26 °C, 16 h photoperiod and 70% relative humidity).

2.2. D. melanogaster strains

Fly strains utilised in this study were maintained on standard food (Bloomington formulation) at 24 °C. The wild type strain Canton-S (#1, wild type) and the A301S strain (#35492, Rdl^{MD-RR}) were sourced from the Bloomington *Drosophila* Stock Center at Indiana University, USA.

2.3. Leaf dip bioassay

Adults were taken from age-structured populations and were aged <10 days old. Rice stems (10 cm cut length) were dipped into the required concentrations of formulated fiprole insecticide (ethiprole SC 200 and fipronil WG 80, Bayer CropScience, Monheim, Germany) for 20 s, air-dried and placed in a plastic specimen tube. Approximately 15 adults were aspirated directly into each tube and sealed with a ventilated lid. A small hole (3 mm diameter) was drilled in the base of each of the tubes, which were then stored vertically in a water bath (submerging only the base of each stem) at 26 °C for 72 h. Mortality was assessed and adults showing no sign of movement were scored as dead. Bioassays consisted of 3 replicates at each concentration. For synergism assays, each insect was treated upon the pronotum with 0.2 μL of 100 mg/L^{-1} piperonyl butoxide (PBO in acetone) (20 ng $adult^{-1}$) and then transferred to rice stems dipped in fipronil. Mortality was assessed at 48 h.

2.4. Genotyping via sequencing

Genomic DNA from individual adults was extracted using 15 μL microlysis plus extraction buffer (Microzone Ltd., Haywards Heath, Sussex, UK) following the manufacturer's recommended protocol for tough cells. A typical PCR (25 μL) contained 0.5 μM of each primer (Table S1), 2 μL extracted DNA, 12.5 μL DreamTaq (Thermo Fisher, Waltham, MA, USA) containing Taq polymerase, 2× PCR buffer and 4 mM $MgCl_2$ (2 mM final concentration). Cycling conditions were 95 °C for 2 min followed by 30 cycles of 95 °C for 30 s, 57 °C for 30 s and 72 °C for 1 min, and a final elongation at 72 °C for 5 min. PCR products was verified by agarose gel electrophoresis prior to PCR cleanup and sequencing which was carried out by Eurofins Genomics (Ebersberg, Germany).

Table 1
Mortalities (%) (± standard error) for all *N. lugens* at two diagnostic doses (LD_{95} and 5 X LD_{95} of the susceptible strain) of ethiprole by leaf-dip bioassay.

Compound	Strain	3 mg L^{-1} (±SE)	15 mg L^{-1} (±SE)
Ethiprole	Bayer-S	100.00 ± nc	100 ± nc
	NI33	6.72 (±3.82)	nt
	NI55	13.89 (±5.69)	8.64 (±4.68)

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Appendix C

Table 2
Dose-response data for *N. lugens* laboratory susceptible and fiprol-resistant strains against ethiprole and fipronil applied as a leaf dip to adults.

Compound	Strain	Generations without selection	LC_{50} [mg L^{-1}]	95% limits	Slope (± SD)	RR
Ethiprole	Bayer-S	174	0.34	0.24–0.44	2.671 ± 0.432	1
	NI33	27	138.3	90.82–198.3	1.32 ± 0.148	406.8
	NI55	18	112.7	54.03–281.8	0.693 ± 0.124	331.5
	NI33-eth	–	>5000	–	–	>14000
	NI55-eth	–	>5000	–	–	>14000
Fipronil	Bayer-S	174	1.16	0.70–1.66	10.858 ± 0.864	1
	NI33	46	37.13	1.06–137.3	1.259 ± 0.453	32
	NI55	29	3.46	0.77–8.21	0.966 ± 0.197	3
	NI33-eth	–	>1000	–	–	>860
	NI55-eth	–	>1000	–	–	>860

Sequence analysis and protein alignments were done with Geneious R8 (Biomatters, Auckland, New Zealand).

2.5. RNA extraction and illumina sequencing

Total RNA was extracted from pooled homogenates of six insects of each of the five strains detailed in this study using the Bioline Isolate RNA Mini Kit (Bioline, London, UK) according to the manufacturer's guidelines. Prior to the RNAseq experiment the quality and quantity of RNA was checked using a NanoDrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Total RNA was used as a template for the generation of barcoded libraries (TrueSeq RNA library preparation, Illumina). Libraries were sequenced by The Genome Analysis Centre (TGAC, Norwich, UK) with replicates multiplexed for sequencing on an Illumina HiSeq 2500 flowcell (100 bp paired end reads) to generate at least 15 million reads per biological replicate. FastQC (version 0.11.2) was used to check the quality of the raw reads obtained.

2.6. SNP calling of RNA-Seq reads aligned to BPH Rdl

Raw reads of each BPH strain were mapped to the BPH *Rdl* reference gene sequence (accession no KX592155). Geneious R8's (Biomatters, Auckland, New Zealand) map to reference function was used with the BPH *Rdl* gene as the reference. Settings were: no gaps, maximum mismatches: 10%, minimum overlap identity: 80%, index word length: 14, maximum ambiguity: 4. All reads that aligned to AA residue 301 and 359 were then assessed for their nucleotide bases.

2.7. Pyrosequencing

For pyrosequencing purposes genomic DNA was extracted from individual BPH adults either using Microlysis-Plus-DNARelease Buffer (Microzone, UK) or QuickExtract Solution (Epicentre, USA) according to the supplier's recommended protocol. *Rdl* gene fragments were amplified by PCR from 50 ng aliquots of gDNA using two primers for the desired target sequence (Table S1; e.g. BPH_Q359E_fw & BPH_Q359E_rev_Btn for *Rdl* Q359E (Fig. S1) designed with Geneious 8 (Biomatters Ltd.) utilizing a partial sequence of the brown plant hopper GABA receptor gene. The pyrosequencing protocol comprised of 40 PCR cycles with 0.67 μM forward and reverse primer (one biotinylated, see Table S1) in 30 μL reaction mixture containing 2× JumpStart Taq ReadyMix (Sigma-Aldrich, Germany) and cycling conditions of 95 °C for 3 min, followed by 40 cycles of 95 °C for 30 s, 57 °C for 30 s and 72 °C for 1 min, and a final incubation at 72 °C for 5 min in a C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Inc.).

In addition, we also analysed the obtained plasmids for sequence correctness at the respective *Rdl* mutation positions by pyrosequencing, i.e. Q359E and A301S. A single PCR was conducted for each mutation site from 50 ng plasmid DNA using two primers (Q395E: BPH_Q359E_Plasmid_fw_Btn and BPH_Q359E_Plasmid_rev; A301S: BPH_A301S_Plasmid_fw_Btn and BPH_A301S_Plasmid_rev, Table S1). The PCR prior to pyrosequencing was carried out in 40 cycles with 0.5 μM forward and biotinylated reverse primer and 2× JumpStart Taq ReadyMix in 30 μL reaction volume and cycling conditions of 95 °C for 3 min, followed by 40 cycles of 95 °C for 30 s, annealing temperature for Q359E PCR 52 °C and for A301S PCR 54 °C, followed by 72 °C for 1 min, and a final incubation at 72 °C for 5 min.

Single strand DNA preparation required for pyrosequencing was done using the PyroMark Q96 Vacuum Workstation (Qiagen) in combination with streptavidin coated beads (Streptavidin Sepharose) to separate the biotinylated strand of the PCR products. The pyrosequencing reactions were carried out according to the manufacturer's instructions utilizing the PyroMark Gold Q96 Reagent Kit (Qiagen) and the respective sequencing primers for genotyping (individual BPH adults: BPH_Q359E_seq; plasmids: BPH_Q359E_Plasmid_seq and BPH_A301S_Plasmid_seq; Table S1). The genotypes were analysed using the supplied PyroMark Q96 ID Software 2.5 (Qiagen). A typical example of the Q359E pyrosequencing results is shown (Fig. S2).

2.8. Preparation of cRNAs encoding N. lugens Rdl variants

Three variants of *N. lugens Rdl* were synthesized and sub-cloned into the expression vector pcDNA3.1(+) by Thermo Fisher Scientific (Life Technologies GmbH, Darmstadt, Germany): wildtype *Rdl* (accession no KX592155), *Rdl*-(A301S) and *Rdl*-(A301S + Q359E). The obtained plasmids were linearized by BbsI digestion according to manufacturer instructions (New England BioLabs Inc., USA), briefly: 20 μg plasmid DNA was incubated with 50 units BbsI for 3 h at 37 °C in a total volume of 100 μL. Subsequently the linearized DNA was purified using Qiagen QIAquick PCR Purification Kit (Qiagen GmbH, Germany). The capped cRNAs were generated using the mMESSAGE mMACHINE T7 transcription kit (ABI-Ambion, USA) and dissolved in RNase-free water at concentrations of 1718 ng/μL (wildtype *Rdl*), 1699 ng/μL (*Rdl*-(A301S)) and 1677 ng/μL (*Rdl*-(A301S + Q359E)).

2.9. Rdl expression and electrophysiological recordings in Xenopus oocytes

Defolliculated oocytes from *Xenopus laevis* in Barth's solution supplemented with gentamycin were received from Ecocyte Bioscience (Castrop-Rauxel, Germany). They were prepared and shipped one day

Fig. 1. Amino acid sequence of TM2 and TM3 (TM regions underlined) from *N. lugens* RDL. The alanine and glutamine residues that are mutated in fiprole resistant strains are highlighted.

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before injection. Oocytes were injected with 75 nL of the prepared cRNA at a concentration of 100 ng/μL. All injections were performed using the automated injection system Roboinject (Multi Channel Systems MCS GmbH, Reutlingen, Germany). Oocytes were then incubated in Barth's solution with gentamycin (20 μg/mL) at 19 °C for 1–3 days. For electrophysiological measurements oocytes were superfused with Normal Frog Ringer (NFR) solution (DiacleanShop, Castrop-Rauxel, Germany) and voltage-clamped at a holding potential of −80 mV using an automated two-electrode voltage clamp Roboocyte set-up (Multi Channel Systems MCS GmbH, Reutlingen, Germany). GABA was dissolved in NFR, whereas stock solutions of test compounds were prepared in DMSO and subsequently diluted with NFR to the desired test concentrations. Antagonist incubations were conducted with slight modifications according to Liu et al. (2015), i.e. antagonist solutions were perfused alone for 60 s after at least four successive GABA applications (EC_{50}), followed by repeated antagonist/GABA (EC_{50}) co-applications at 30 s intervals. Electrophysiological recordings were analysed using the software package Roboocyte V2.2.0 (Multi Channel Systems MCS GmbH, Reutlingen, Germany) and EC_{50}/IC_{50}-values were calculated from plotting normalized responses as a function of compound concentration using GraphPad Prism Software 5.03 (Graphpad Software, Inc., USA).

2.10. D. melanogaster insecticide bioassays

3–5 day old adult females were used in insecticide bioassays to assess the susceptibility of different fly strains to the technical compounds, ethiprole and fipronil (Sigma Aldrich, St. Louis, MO, USA). The flies were subjected to the insecticide in a contact/feeding bioassay. The full bioassay method is described in a previous paper [29]. The raw data was corrected for control mortality using Abbott's formula [30] and lethal concenctration values LC_{50} and LC_{95} were calculated by probit analysis using the GenStat® (2014, 17th Edition, ©VSN International Ltd., Hemel Hempstead, UK) statistical system.

2.11. Modelling

Protein modelling of the RDL *Nilaparvata* sequence was performed using the Orchestrar suite within the Certara software package Sybylx 2.1.1 (Certara L.P., St. Louis, MO). The crystal structure of the GluCl channel of *Caenorhabditis elegans* (PDB-Id: 3RHW) served as a template for the construction of the monomeric *Nilaparvata* homology model. Overall amino acid sequence identity between the monomers of the two species was 38.6%. The pentameric arrangement was realized by an iterative fit to each of the five subunits of the original crystal structure, followed by a subsequent energy minimization to remove any unwanted contacts and conformational distension from the complete model construction.

2.12. Database submission

Sequence data used in this study have been deposited at the National Center for Biotechnology Information as follows:

BioProject (accession no PRJNA331084).

Table 3
Genotypes via Sanger sequencing of *N. lugens* strains for A301S and Q359E.

Population	A301S genotype (%)			Q359E genotype (%)		
	RR	SR	SS	RR	SR	SS
Bayer-S	0	0	100	0	0	100
NI55	12.5	55	32.5	7.14	35.71	57.14
NI55-eth	100	0	0	86.84	13.16	0

(NI55 A301S N = 40, NI55-eth A301S N = 40, NI55 Q359E N = 28 and NI55-eth Q359E N = 38).

Table 4
SNP calling via RNA-Seq of *N. lugens* strains for A301S and Q359E.

		NI33		NI33-eth		NI55		NI55-eth	
		No. reads	%	No. reads	%	No. reads	%	No. reads	%
A301S	Total reads	20	–	34	–	10	–	18	–
	G (WT)	17	85	0	0	8	80	0	0
	T (Mut)	3	15	34	100	2	20	18	100
Q359E	Total reads	25	–	30	–	11	–	23	–
	C (WT)	25	100	30	100	8	72.73	1	4.35
	G (Mut)	0	0	0	0	3	27.27	22	95.65

BioSample (accession numbers SAMN05437238, SAMN05437239, SAMN05437240, SAMN05437241, SAMN05437242).

Run (accession no SRP079631).

3. Results

3.1. Fiprole bioassays

Diagnostic dose bioassays with ethiprole (Table 1), were performed on NI33 and NI55 soon after field collection, with high levels of resistance seen compared to the susceptible Bayer-S strain, previously reported in [5]. Log-dose probit-mortality data obtained from leaf dip bioassays are presented in detail (Table 2). Ethiprole resistance of the unselected NI33 and NI55 populations was 406-fold and 331-fold respectively compared to the lab susceptible strain Bayer-S. Selection of these strains with ethiprole (NI33-eth and NI55-eth) resulted in a drastic increase in resistance of > 14,000-fold. With a resistance ratio of 32-fold NI33's level of resistance to fipronil was markedly lower compared with its ethiprole resistance. The same observation, but much more profound, was made for NI55, which displays only a 3-fold resistance to fipronil. The two selected strains, in contrast, demonstrate similarly high levels of resistance to fipronil, with approximately 860-fold resistance against Bayer-S.

3.2. Genotyping A301S and Q359E via sanger sequencing

The mutations analysed in this study are shown (Fig.1). All strains were analysed for the presence of the A301S mutation by Sanger sequencing of an amplified 257 bp sequence from genomic DNA. Genotyping of A301S in Bayer-S confirmed the wild type genotype (Table 3). NI55 on the other hand displayed a mix of genotypes, with only 12.5% of insects homozygous for A301S and 32.5% homozygous for the wildtype genotype. 100% of insects analysed from NI55-eth carried the A301S mutation in the homozygous form. A novel mutation, Q359E, was also identified but only in NI55 and NI55-eth, with all other strains being 100% homozygous for the wildtype genotype at this AA residue. NI55 displayed 7% of individuals homozygous for the Q359E mutation, with 57% of insects homozygous for the wildtype genotype. However, 87% of individuals were homozygous for the Q359E mutation in NI55-eth, while the remaining 13% were heterozygous. Since the A301S mutation reached fixation in NI55-eth it can be concluded that there are two A301S alleles present in that strain, one with and one without the Q359E mutation.

Table 5
Pyrosequencing of Q359E in two populations of *N. lugens*.

Population	Allele frequency		
	Q	Q/E	E
NI55	0.69	0.28	0.03
NI55-eth	0.02	0.24	0.74

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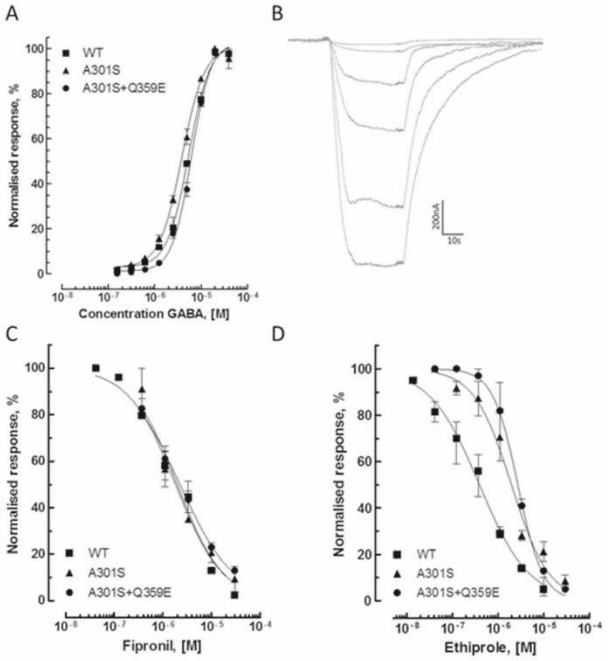

Fig. 2. Effect of GABA and fiprole antagonists on GABA-induced currents in *N. lugens* RDL receptors functionally expressed in *Xenopus* oocytes. (A) GABA concentration-response curves on wildtype (WT) and mutant RDL variants carrying an A301S and A301S + Q359E amino acid substitution, respectively. Data are mean values ± SEM (n = 3); (B) Typical example of electrophysiological oocyte recordings showing the concentration-dependent action of GABA (10, 5, 2.5, 1.25, 0.625, 0.313 and 0.156 μM) on functionally expressed receptors (*Rdl* A301S). (C, D) Antagonist concentration-response curves for fipronil and ethiprole on three different RDL variants. The responses were normalized relative to the currents induced by 5 μM GABA for each receptor variant. Data are mean values ± SEM of 3–5 independent recordings.

3.3. SNP calling of A301S and Q359E via RNA-Seq

For all the strains, RNA-Seq reads were mapped against the BPH *Rdl* nucleotide sequence to observe any non-synonymous mutations, of which there were two: A301S and Q359E. Bayer-S SNP calling of A301S, displayed 100% of reads containing the wild type genotype at AA residue 301 (Table 4). NI33 and NI55 exhibited 85% and 80% of reads with the wild type genotype respectively. While for the NI33-eth and NI55-eth populations, 100% of reads contained the A301S mutation. In agreement with Sanger sequencing, the SNP calling of RNAseq data showed that the Q359E mutation was only found in NI55 and NI55-eth (Table 4), with 96% of NI55-eth reads containing the Q359E mutation, compared to 27% for NI55.

3.4. Genotyping of Q359E via pyrosequencing

Ninety-six insects each of NI55 and NI55-eth were assessed for genotype at the 359 position (Table 5). NI55 (unselected) showed 69% of individuals homozygous for Q, while only 3% homozygous for E, with the remaining individuals heterozygous for Q/E. NI55-eth (selected) displayed 2% of individuals homozygous for Q, while 74% of individuals

Table 6
Log-dose probit mortality data for fiproles against *Drosophila melanogaster* strains.

Compound	Strain	LC_{50} [mg L^{-1}]	95% CL	LC_{95} [mg L^{-1}]	95% CL	Slope (± SD)	Resistance ratio LC_{50}	Resistance ratio LC_{95}
Ethiprole	Canton-S	5.73	4.77–6.77	22.39	17.14–33.08	2.777 ± 0.238	1	1
	RDL-MD-RR	>25000	–	>25000	–	–	>4300	>1100
Fipronil	Canton-S	1.27	0.77–1.85	9.04	5.29–25.55	1.931 ± 0.333	1	1
	RDL-MD-RR	8.82	5.34–13.7	62.36	33.11–238.1	1.936 ± 0.363	6.9	6.9

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Table 7
Mortalities (%) of ethiprole selected populations to fipronil after application of PBO.

Strain	100 mg L^{-1}	500 mg L^{-1}
NI33-eth	20	16
NI55-eth	10	24

were homozygous for E, with the remaining individuals heterozygous for Q/E.

3.5. Sensitivity of wildtype and mutant N. lugens Rdl receptors to GABA, ethiprole and fipronil

All three cRNA variants resulted in functional GABA-gated chloride channels when injected in *Xenopus* oocytes, i.e. RDL wildtype (WT), RDL-(A301S) and RDL-(A301S + Q359E). Concentration dependent inward currents were obtained in voltage-clamp recordings in response to bath-applied GABA indicating the functional expression of homomeric RDL receptors in oocytes (Fig. 2A and B). The agonist pEC_{50}-values calculated from the fitted curves were 5.43 ± 0.02, 3.90 ± 0.03 and 5.21 ± 0.02 for RDL wildtype, RDL-(A301S) and RDL-(A301S + Q359E), respectively. Both fipronil and ethiprole reduced the response of the three RDL subtypes to GABA (measured at EC_{50}) in a concentration dependent manner (Fig. 2C and D). No significant difference in the antagonistic action of fipronil was measured between RDL subtypes: RDL wildtype, pIC_{50} 5.74 ± 0.06; RDL-(A301S), pIC_{50} 5.70 ± 0.08; and RDL-(A301S + Q359E), pIC_{50} 5.65 ± 0.08. However, for ethiprole significant differences in antagonistic action were obtained between RDL wildtype (pIC50 6.41 ± 0.05) and the two mutated subtypes, RDL-(A301S) (pIC_{50} 5.70 ± 0.06) and RDL-(A301S + Q359E) (pIC_{50} 5.56 ± 0.03).

3.6. D. melanogaster fiprole bioassays

The RDL-MD-RR (carrying *Rdl* A301S) strain displayed high levels of resistance to ethiprole with a resistance ratio > 4000 fold based on the LC_{50} when compared with the wildtype strain, Canton-S (Table 6). Against fipronil the RDL-MD-RR strain had a resistance ratio of only 6.9-fold.

3.7. Synergist bioassays with PBO + fipronil

Synergistic bioassays were conducted with PBO on the highly fipronil resistant populations NI33-eth and NI55-eth to assess whether P450 monooxygenases (and esterases) could be potentially contributing to the resistance phenotype observed. Fipronil mortality of both populations was under 25% against a concentration of 500 mg L^{-1} (Table 7) indicating that the majority of the individuals of both strains is unaffected by the application of PBO prior to exposure to fipronil.

4. Discussion

To date, the molecular basis of ethiprole resistance in *N. lugens* has remained unclear. A previous study linked esterase activity, and to a lesser extent P450s activity, to ethiprole resistance in *N. lugens* in central Thailand, based on the separate application of PBO, triphenyl phosphate and diethyl maleate as synergists prior to ethiprole exposure [27]. However, to date, no mutation(s) in the non-competitive antagonist binding site of RDL has been implicated in resistance to ethiprole. In the case of fipronil resistance, a potential novel mechanism of resistance was very recently implicated in a laboratory selected strain of *N. lugens* (see Introduction), but was not observed at sufficient frequency to cause resistance in field populations [24].

In this study, we identified two mutations in *Rdl* associated with phenylpyrazole resistance in two field strains. Both strains, NI33 (Vietnam) and NI55 (India) exhibited high levels of resistance to ethiprole, despite a long period of non-selection (27 and 18 generations respectively). When these strains were exposed to continuous ethiprole selection, their resistance markedly increased compared to the non-selected populations. We identified two mutations in these strains; the first was the previously reported A301S mutation [24], which was observed at low frequency in both parental field strains but rapidly rose in frequency and became fixed under ethiprole-selection. We further identified a novel mutation, Q359E, in one of the strains that also increased in frequency under selection. Subsequent functional analysis of the role of these mutations in resistance to fipronil and ethiprole, provided several lines of evidence to support a causal role of the A301S mutation in resistance to ethiprole.

Firstly, expression of recombinant wild-type and A301S RDL receptors in *Xenopus* oocytes followed by electrophysiological assays showed that presence of the A301S mutation reduces the sensitivity of the

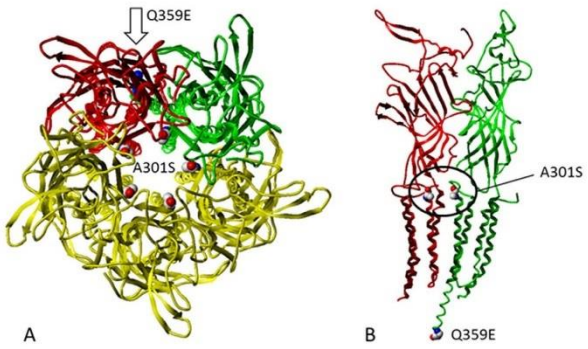

Fig. 3. A) Top-view of the RDL GABA-R homo-pentamer (*NI* RDL homology model based on 3RHW) showing three subunits in yellow, one in green and red, respectively. The mutation site A301S is located in the middle of the M2 transmembrane helices forming the channel pore. The other mutation site Q359E is located intracellularly at the end of helix M3 outside the pore region (indicated by an arrow). B) Side-view showing two of the RDL subunits and the location of the mutation site A301S in transmembrane pore helix M2, whereas mutation site Q359E is located >40 Å from this residue (the helical structure of the domain is proposed as amino acid positions 337–428 are missing in the modelling template 3RHW). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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Appendix C

receptor to ethiprole 8–10-fold compared to wild-type providing strong evidence of a role in vitro. Further in vivo evidence of the role of this mutation in ethiprole resistance was also provided by insecticide bioassays of a *D. melanogaster* line with the same mutation, which exhibited 4000-fold resistance to ethiprole in comparison to a strain without the mutation.

In contrast to our findings with ethiprole very limited evidence was seen for a causal role of the A301S mutation in resistance to fipronil. In electrophysiological assays the recombinant A301S RDL receptor showed no significant shift in sensitivity to fipronil, with a response broadly similar to that of the wild type. A low level of resistance to fipronil was seen in the *D. melanogaster* line with the A301S mutation (around 7-fold compared to Canton-S). This result is similar to a recently reported study (13.8-fold) [16], on the same strain. A different strain of *D. melanogaster* strain carrying the same mutation was previously reported to show 73-fold resistance to fipronil [31], however, such high levels of resistance to fipronil were not apparent in our study or that carried out previously by Remnant et al. 2014.

As detailed above a second mutation, Q359E, was also observed in the NI55 strain from India at low frequency but increased to high frequency upon ethiprole selection. All insects with Q359E carried it in combination with A301S. Since this mutation is never seen in isolation in the selected population, we decided to focus our analysis on the double mutant variant (A301S + Q359E) via electrophysiology to assess the effect of Q359E in tandem with A301S.

Our data suggest that in comparison to A301S, the Q359E mutation plays no direct role in resistance to either ethiprole or fipronil with double mutant receptors displaying the same level of sensitivity to both compounds as the single mutant receptor (A301S). A model of the *N. lugens* RDL channel places the Q359E mutation at least >40 Å away from the key A301S residue which could be a potential reason for its lack of direct impact (Fig. 3). However, a previous study using the Drosophila Genetic Reference Panel (DGRP) lines identified three fipronil resistant strains (A301S + T350S, A301S + T360I and A/S301 + M/I360) demonstrating the ability of multiple mutations in the *Rdl* to cause fipronil insensitivity [16]. Duplication at the *Rdl* locus has been described, and demonstrated the ability to accrue resistance mutations but maintain wildtype functionality in this insecticide target site [32]. In this study the Q359E mutation has only been tested in vitro and it would be interesting in future to examine its role in vivo in either *N. lugens* or *Drosophila* by using transgenic approaches such as the CRISPR/Cas system [29].

In the light of our results there are two possible explanations for the increase in frequency of the Q359E mutation under ethiprole selection. Firstly, it is a random polymorphism that because of its close proximity to A301S has hitchhiked to high frequency due to the physical linkage of the two mutations and the adaptive advantage of A301S. Secondly, this mutation, while not directly contributing to ethiprole resistance, may have a fitness benefit, to *N. lugens* individuals that carry this mutation in combination with A301S. For example, the Q359E mutation might act as a compensatory mutation for A301S as has been recently claimed for the R299Q substitution (see Introduction). Our results do not support this idea as recombinant receptors with A301S alone and A301S + Q359E show the same affinity for the native ligand GABA. Furthermore, A301S has been shown to persist in other insect species at high frequency in the absence of insecticide selection [33], suggesting it may have a minimal fitness penalty.

The A301S mutation was one of the first target-site resistance mutations to be described in insects and has since appeared in a wide array of different insect species [32]. Originally described as the primary mechanisms of resistance to cyclodienes, it has also been linked with low level cross-resistance to fipronil [16,17]. The effect of A301S in relation to cyclodiene resistance is two-fold, it reduces insecticide binding and destabilises the antagonist favoured structure of the RDL channel [34]. Surprisingly, this mutation has never been previously implicated in ethiprole resistance. Fipronil and ethiprole are highly structurally similar (Fig. S3) and so it is surprising that the A301S mutation can provide such effective resistance against ethiprole, but not to the same extent against fipronil.

The extremely high resistance levels seen in BPH strains selected with ethiprole, cannot be completely explained by the *Rdl* A301S mutation. The difference between wild-type and A301S RDL constructs in the voltage clamp recordings, was not enough to be wholly responsible for the resistance described in Table 2. Therefore, there must be another mechanism of resistance capable of causing resistance to ethiprole within the BPH populations tested here. We hypothesise that the unknown fipronil resistance mechanism (discussed later) could cause cross resistance to ethiprole, and therefore explain the very high levels of resistance in these BPH strains.

Zhang et al. recent study on R299Q and A301S mutations in RDL and their correlation with fipronil resistance [24], has similarities with our study. The finding of a novel mutation existing only in tandem with the A301S mutation is a key finding. However, the new mutation they describe (R299Q) appears to increase resistance to fipronil when combined with A301S, further than A301S by itself. Although we find a novel mutation, it does not have the same direct impact as R299Q. Similar to Zhang et al. we also find that the RDL mutations we analysed in this study are not the main mechanism of resistance to fipronil in the BPH populations tested.

The lack of impact of either of the RDL mutations against fipronil led us to carry out tests with the P450 and esterase inhibitor PBO to explore if these enzyme systems are involved in resistance to this compound. In this regard, recent research has used the same approach to implicate metabolic mechanisms in resistance to fipronil in *N. lugens* [24]. In our study the application of PBO had no noticeable impact on the fipronil resistance of the resistant populations, NI33-eth and NI55-eth suggesting P450s and/or esterases are either not involved in resistance or play a minor role. However, in contrast to our study Zhang et al. applied a mixture of synergists (PBO, triphenyl phosphate and diethyl maleate), so it is possible that other enzyme systems that are inhibited by triphenyl phosphate and/or diethyl maleate are involved in resistance such as glutathione S-transferases [24].

An interesting observation from our selection experiments was that the selection of the NI33 and NI55 strains with ethiprole also increased resistance to fipronil. Despite this observation, our data clearly suggest that the mutations analysed in this study are not the explanation for the increased fipronil resistance in the NI33-eth and NI55-eth strains and the molecular basis of fipronil resistance in these strains requires further investigation. However, this finding is of field relevance as it suggests that fipronil would not be a viable substitute for ethiprole, if ethiprole was no longer able to control *N. lugens* within economic thresholds.

In conclusion, two mutations (A301S and Q359E) were identified in the *Rdl* gene of *N. lugens* and assessed for their potential role in resistance to fiproles. Our results indicate that the common A301S mutation confers resistance to ethiprole, a widely used insecticide for the control of brown planthopper. However, neither this mutation nor the novel mutation Q359E causes significant resistance to fipronil based on the in vitro and in vivo studies conducted here. Our finding that selection with ethiprole also selects for cross-resistance to fipronil is relevant to the future application of these insecticides in the field and the design and implementation of resistance management programmes.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.pestbp.2017.01.007.

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A CRISPR/Cas9 mediated point mutation in the alpha 6 subunit of the nicotinic acetylcholine receptor confers resistance to spinosad in *Drosophila melanogaster*

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ABSTRACT

Spinosad, a widely used and economically important insecticide, targets the nicotinic acetylcholine receptor (nAChRs) of the insect nervous system. Several studies have associated loss of function mutations in the insect nAChR α6 subunit with resistance to spinosad, and in the process identified this particular subunit as the specific target site. More recently a single non-synonymous point mutation, that does not result in loss of function, was identified in spinosad resistant strains of three insect species that results in an amino acid substitution (G275E) of the nAChR α6 subunit. The causal role of this mutation has been called into question as, to date, functional evidence proving its involvement in resistance has been limited to the study of vertebrate receptors. Here we use the CRISPR/Cas9 gene editing platform to introduce the G275E mutation into the nAChR α6 subunit of *Drosophila melanogaster*. Reverse transcriptase-PCR and sequencing confirmed the presence of the mutation in *Dα6* transcripts of mutant flies and verified that it does not disrupt the normal splicing of the two exons in close vicinity to the mutation site. A marked decrease in sensitivity to spinosad (66-fold) was observed in flies with the mutation compared to flies of the same genetic background minus the mutation, clearly demonstrating the functional role of this amino acid substitution in resistance to spinosad. Although the resistance levels observed are 4.7-fold lower than exhibited by a fly strain with a null mutation of *Dα6*, they are nevertheless predicated to be sufficient to result in resistance to spinosad at recommended field rates. Reciprocal crossings with susceptible fly strains followed by spinosad bioassays revealed G275E is inherited as an incompletely recessive trait, thus resembling the mode of inheritance described for this mutation in the western flower thrips, *Frankliniella occidentalis*. This study both resolves a debate on the functional significance of a target-site mutation and provides an example of how recent advances in genome editing can be harnessed to study insecticide resistance.

1. Introduction

Insecticide resistance is an exceptional example of rapid adaptive evolution and has provided a range of insights into the diversity of genetic alterations that occur in response to novel selective pressures. A common mechanism of insect resistance to insecticides, termed 'target-site resistance' involves alterations (mutations) in the insecticide target protein that reduce its sensitivity to insecticide. Target-site resistance most frequently involves point mutations at select positions in the target receptor as small changes to proteins are least likely to disrupt their, usually important, native function (ffrench-Constant et al., 1998).

The nicotinic acetylcholine receptor (nAChR) α6 subunit is a rare example of an insecticide target-site that can tolerate more radical alterations as it appears to be a redundant target (Perry et al., 2007). Insect α6-containing receptors are the target of spinosad, a macrocyclic lactone bio-insecticide derived from secondary

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metabolites of the soil bacteria *Saccharopolyspora spinosa*. Several lines of research indicate that spinosad binds at a site distinct from the neonicotinoid insecticides one exerting its effect through an allosteric mechanism (Orr et al., 2009; Puinean et al., 2013; Salgado and Saar, 2004).

The first resistance-conferring mutation described in the gene encoding this subunit was a null mutation of *Dα6*, in the fruit fly, *Drosophila melanogaster*, which was found to result in >1000-fold resistance to spinosad (Perry et al., 2007). Significantly, mutant flies were viable and displayed no obvious fitness deficit. Based on this finding Perry et al. predicted that loss of function mutations in *Dα6* orthologues may lead to spinosad resistance in field populations of insect pests (Perry et al., 2007). This prediction has held true with a range of genetic alterations in nAChR *α6* now described in several insect crop pests that result in truncated non-functional proteins. For example, several mutations resulting in mis-splicing and premature stop codons in nAChR α6 transcripts are associated with spinosad resistance in the diamondback moth, *Plutella xylostella*, and the oriental fruit fly *Bactrocera dorsalis* (Baxter et al., 2010; Hsu et al., 2012; Rinkevich et al., 2010).

Recently, however, spinosad resistance in several insect pest species has been associated with the same non-synonymous point mutation in exon 9 of the α6 nAChR that does not result in loss of function (Bao et al., 2014; Puinean et al., 2013; Silva et al., 2016). This mutation was initially described in western flower thrips, *Frankliniella occidentalis* (Puinean et al., 2013), and results in the replacement of a glycine (G) residue at position 275 observed in susceptible strains with a glutamic acid (E) in resistant strains. The same substitution was subsequently described in spinosad resistant melon thrips, *Thrips palmi* (Bao et al., 2014), and, very recently, tomato leafminer, *Tuta absoluta* (Silva et al., 2016).

The causal role of this mutation in resistance was recently questioned by Hou et al., after these authors characterized the nAChR α6 from three susceptible and two spinosad resistant strains of *F. occidentalis* from China and the USA and observed no difference in the cDNA sequences of resistant and susceptible thrips (Hou et al., 2014).

Functional validation of mutations in insect nAChRs, such as G275E, has been hampered by difficulties encountered in their expression in heterologous systems. Indeed, as a surrogate, Puinean et al. examined the potential effects of the G275E using human nAChR α7, a model receptor that readily forms functional homomeric receptors when expressed in heterologous systems (Puinean et al., 2013). Expression of the analogous mutation (A275E) in human α7 in *Xenopus oocytes* was found to abolish the modulatory effects of spinosad but had no significant effect upon activation by the natural ligand acetylcholine (Puinean et al., 2013). Although this evidence supports a causal role for the mutation, no functional validation of G275E in an insect system has been performed to date.

The type II clustered regular interspersed short palindromic repeat (CRISPR)/associated protein-9 nuclease (Cas9) system has recently emerged as an efficient tool to introduce precise, targeted changes to the genome of living cells. The CRISPR/Cas9 system exploits the RNA-guided endonuclease function of Cas9 to introduce double-stand breaks (DSBs) at defined loci that are then repaired by either nonhomologous end joining (NHEJ) or homology-directed repair (HDR). To introduce single nucleotide replacements in target genes HDR is exploited to repair DSBs by providing homologous sequence from a donor template such as a ssOligo or plasmid. CRISPR/Cas9-mediated editing of the genome of *D. melanogaster* has now been reported (Port et al., 2014) and the first use of this technology to introduce a resistant mutation into a controlled genetic background has also recently been described (Somers et al., 2015).

Here we describe the use of the CRISPR/Cas9 system to introduce the G275E mutation into *D. melanogaster* and demonstrate the causal role of this amino acid replacement in resistance to spinosad.

2. Material and methods

2.1. D. melanogaster strains

Fly strains described in this study were maintained on standard food (Bloomington formulation) at 24 °C. Fly strains deficient for DNA ligase 4 (#28877, genotype w^{1118} $Lig4^{169}$), expressing endonuclease Cas9 (#51324, genotype w^{1118}; PBac{y[+mDint2] = vas-Cas9}VK00027), deficient for the alpha6 subunit of the nAChR (#556, genotype w*; Df(2L)s1402, P{w[+mC] = lacW}s1402/CyO) and throughout the manuscript referred to as 'Dα6 KO', as well as the wildtype strain Canton-S (#1, wildtype) were sourced from the Bloomington Drosophila Stock Center at Indiana University, USA. The lig4 deficient strain and the Cas9 expressing strain were crossed and consecutive PCR assisted sibling mating allowed the rescue of a strain homozygous for both traits (genotype w^{1118} $Lig4^{169}$; Bac{y[+mDint2] = vas-Cas9}VK00027), hereafter called 'lig4 KO Cas9'.

2.2. gRNA design, template oligo and plasmid construction

The gRNA was designed using the online platform http://www.flyrnai.org/crispr2/ (Housden et al., 2015). A region spanning ~250 bp either side (>2L:9798031-978511) of the position of the desired point mutation was specified for the design. Based on the number of predicted off-targets a gRNA (>2L 2L:9798305.9798324 (- strand) AATTTCGCACCTAAATCCTT) was chosen as this was the only gRNA predicted to have no off-targets in combination with the predicted cutting site in close proximity to the nt position of the desired mutation (2L:9798305/9798306) (Fig. 1A). A gRNA expression plasmid was generated by cloning annealed gRNA oligonucleotides (Table 1) into the pCFD3: U6:3-gRNA plasmid (addgene #49410) as described elsewhere (Port et al., 2014). A single stranded oligonucleotide of 110 nt in size (template ssOligo) was designed to serve as a template for HDR following the Cas9 induced double strand break. The template ssOligo was designed with a dinucleotide polymorphism (Fig. 1B) which when incorporated into the genome would result in an alternate codon encoding glutamatic acid (E) instead of the native glycine (G) at AA position 275 of Dα6 (accession number NT_033779, AA count differs by 26 AA as position 275 refers to the protein after cleavage of the signal peptide). The template also contained a single nucleotide polymorphism (SNP) corresponding to intronic sequence just upstream of the above mutation site to prevent re-cleavage from Cas9 after incorporation (Fig. 1B).

2.3. Embryo injections and rescue of CRISPR mediated mutations

Embryos were collected from lig4 KO Cas9 flies and injections were carried out on an inverted microscope (eclipse Ti–U Nikon, Japan) equipped with a 10×/0.25 lens, 10×/22 eyepiece and fluorescence illumination. The injection mix comprised 0.5× phosphate buffer (pH 6.8, 0.05 mM sodium phosphate, 2.5 mM KCL) containing 200 ng μl^{-1} gRNA expression plasmid, 1 $\mu g\ \mu l^{-1}$ template ssOligo and 200 mg L^{-1} fluorescein sodium salt to improve the monitoring of injections. The mix was delivered by a micromanipulation set-up consisting of a motorised micromanipulator TransferMan NK2 (Eppendorf, Hamburg, Germany) and a Femto Jet express microinjector (Eppendorf, Hamburg, Germany). Injection needles were prepared according to Miller et al. (2002) and injections into non-dechorionated embryos was carried out

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Fig. 1. Dα6 exon organisation, gRNA target site and HDR template. A) Exon organisation, white arrows indicating alternative exon 3a/b and 8a/b (top) and 20 nt CRISPR/Cas9 target site as indicated by the large arrow above the sequence followed by the 'ngg' PAM motif (bottom). B) Nucleotide alignment of the 110 nt HDR template with Dα6. The single C/G substitution prevents re-cleavage of Cas9 through a mismatch in the gRNA seed sequence (last 12 nt of gRNA) and the double substitution GC/AA introduces a codon substitution from glycine (G) to glutamatic acid (E).

Table 1
Oligonucleotide sequences for PCR, for cloning into gRNA expression plasmids and template ssOligo for the manipulation of homology directed repair (HDR) in the germline of *D. melanogaster*.

Sequence name	Sequence 5′–3′	5′ overhang
gRNA oligo forward	AATTTCGCACCTAAATCCTT	GTCG
gRNA oligo reverse	AAGGATTTAGGTGCGAAATT	AAAC
G275E ssOligo	TACTGTCAGCACCACCGACGAGGCGACCATGAACATGATGCAATTGAAGTAGGTT TCTAAGCATTTAGGTGCGAAATTTTGGGTTTGCGGATTGCGGATGGCAGTTTGTG	
a6 gDNA PCR F	ATTTTGAGAGACCCCGGAGC	
a6 gDNA/cDNA R	ATATTGTGTGCCGGAAGTCGT	
a6 gDNA seq	ATTGTGTGCCGGAAGTCGTC	
a6 cDNA F1	TGGCACGTATCACACCAACA	
a6 cDNA F2	CATGTACAACAGCGCGGATG	

according to Miller et al. (2002) with modifications described previously (Arnoult et al., 2013). Emerging flies were crossed to flies of the Dα6 deficient strain, Dα6 KO with the curly wing phenotype (Cy), as we expected both knock-outs as well as the point mutation to be a recessive trait based on previous reports regarding spinosad resistance conferred by this gene (Perry et al., 2007; Puinean et al., 2013). Crossings were performed under spinosad selection pressure subjecting the flies to standard media to which spinosad was added to reach a final concentration of 250 μl L^{-1} active ingredient. This concentration does not affect adult flies but is sufficient to prevent the development of susceptible flies in the next generation (see Supplementary Fig. 1). Developing flies were screened for Cy and Cy males were crossed to virgin Cy Dα6 KO females. 5 days after the crossings were set up the males were retrieved for PCR and sequencing and the females were discarded. The emerging flies were again screened for curly wings and sibling mating was set up to select against Cy in the next generation.

2.4. PCR analysis and sequencing

DNA was extracted from single adult flies using 20 μl microlysis plus extraction buffer (Microzone Ltd., Haywards Heath, Sussex, UK) following the manufacturer's recommended protocol for tough cells. A typical PCR (20 μl) contained 0.5 μM of each primer (Table 1), 2 μl extracted DNA, 10 μl DreamTaq (Thermo Fisher, Waltham, MA, USA) containing Taq polymerase, 2× PCR buffer and 4 mM $MgCl_2$ (2 mM final concentration). Cycling conditions were 95 °C for 2 min followed by 35 cycles of 95 °C for 20sec, 57 °C for 20sec and 72 °C for 1 min, and a final elongation at 72 °C for 5 min. To verify the mutation at the level of the transcript total RNA was extracted from pools of 5 adults using the Isolate II RNA mini kit (Bioline, London, UK) following the manufacturer's protocol. cDNA was transcribed using a cDNA synthesis kit (PCRBIO, London, UK) and 1 μl was subsequently used in a PCR reaction using cycling conditions and master mix as described above but using a nested approach. For the primary PCR primer 'a6 cDNA F1' (Table 1) was used in combination with 'a6 gDNA/cDNA R' with 20 cycles of thermocycling and 0.5 μl of the primary reaction was subsequently used as a template in the secondary PCR with the primers 'a6 cDNA F1' and 'a6 gDNA/cDNA R' with 15 cycles of thermocycling. PCR products were verified by agarose gel electrophoresis prior to PCR cleanup and sequencing which was carried out by Eurofins Genomics (Ebersberg, Germany). Sequence analysis and protein alignments were done with Geneious R8 (Biomatters, Auckland, New Zealand).

2.5. Insecticide bioassays and statistical analysis

3–5 day old adult females were used in insecticide bioassays to assess the susceptibility of different fly strains and crossings to the commercial formulation of spinosad (Conserve®, 11.6% spinosad in SC formulation, Dow Agrosciences, Indianapolis, IN, USA). The flies were subjected to the insecticide in a contact/feeding bioassay. Standard Drosophila vials (#789001, Dutscher Scientific, Brentwood, Essex, UK) were filled with agar solution (4 ml/vial) containing 2% w/v agar (Dutscher Scientific, #789150), 1.2% w/v food grade sucrose and 0.4% v/v glacial acetic acid (Sigma Aldrich, St. Louis, MO, USA). A 5000 mg L^{-1} spinosad stock solution was prepared by adding 43.1 µl Conserve® per ml $^{-1}$ tap water and a 5-fold serial dilution was prepared to achieve a concentration range from 5000 mg L^{-1} to 0.32 mg L^{-1} spinosad in tap water. 18 h prior to bioassay the agar vials were spread with 100 µl of spinosad solution and vortexed vigorously. For each concentration vials were prepared in triplicate for each fly strain. Flies were anaesthesised with CO_2 and 10 female flies added to each vial. The vials were kept upside down until all flies became active to avoid flies getting trapped in agar. The bioassay was assessed after 48 h, dead flies as well as seriously affected flies i.e. those displaying no coordinated movement, that were unable to walk up the vial, or unable to get to their feet were cumulatively scored as 'affected'. The raw data was corrected for control mortality using Abbott's formula (Abbott, 1925) and lethal concentrations LC_{50} and LC_{95} were calculated by probit analysis using the Polo Plus software v.1 (LeOra Software, Berkeley, CA, USA). The mode of inheritance was calculated according to Stone applying the respective LC values (Stone, 1968). Non-linear log dose-response curves were generated in Graphpad Prism 6.07 (Graphpad Software Inc., La Jolla, CA, USA).

3. Results

3.1. CRISPR mediated G275E replacement in D. melanogaster

Approximately 250 embryos were injected with a gRNA plasmid and a single stranded oligonucleotide to serve as a template for HDR. The 41 flies that developed from injected embryos (~16%) were crossed to Dα6 KO flies in vials containing spinosad supplemented media (see Material and Methods) in order to kill progeny carrying the native Dα6 subunit that are highly susceptible to spinosad. 10 of those crossings produced viable offspring (~25%). 100 Cy males were backcrossed to Cy virgin Dα6 KO females and analysed by PCR and direct sequencing 5 days after the crossing. Sequencing of the genomic loci of interest revealed progeny with a variety of INDELS and others with the desired nucleotide editing. In the case of the former, the various insertions and deletions resulted in gene knock outs either by the introduction of premature stop codons, frame shifts or by loss of splice recognition sites (AG). In the case of the targeted nucleotide replacement all three desired point mutations were observed at the correct positions with the two in exon 9 of the *Dα6* gene conferring the G275E replacement (for representative sequencing traces see Fig. 2). All offspring tested by PCR and sequencing exhibited mutations around the CRISPR/Cas9 target site and the ratio of INDEL mutations mediated by NEJR to HDR mediated insertion of the oligo template was 40:60 (based on 85 viable crossings and PCR reactions). Sequencing of cDNA prepared from CRISPR mutated flies verified the introduced point mutations had not disrupted the normal splicing of the *Dα6* mRNA transcript with the introduced G275E mutation present in transcripts with either exon 8a or 8b, as indicated by the clean peaks for the altered nucleotides in exon 9 and the double peaks present in exon 8 where the two splice forms vary (Fig. 3).

3.2. The impact of G275E on the susceptibility of D. melanogaster to spinosad in comparison to Dα6 knock out

The Dα6 G275E substitution reduces susceptibility significantly when compared to the wild type strain Canton-S and the non-modified strain lig4 KO Cas9 which was the genetic background into which the mutation was introduced. The resistance ratio based on LC_{95} values is ~66-fold (Table 2 and Fig. 4). Furthermore, two additional independent lines homozygous for the Dα6 G275E substitution were tested and showed a comparable reduced sensitivity to spinosad (Supplementary Table 1). In comparison the Dα6 KO strain deficient for the Dα6 gene exhibited a resistance factor of 311-fold. This is lower than that reported previously by Perry et al. (2007) and likely results from difference in bioassay methodology used in the two studies. Reciprocal crossing with the

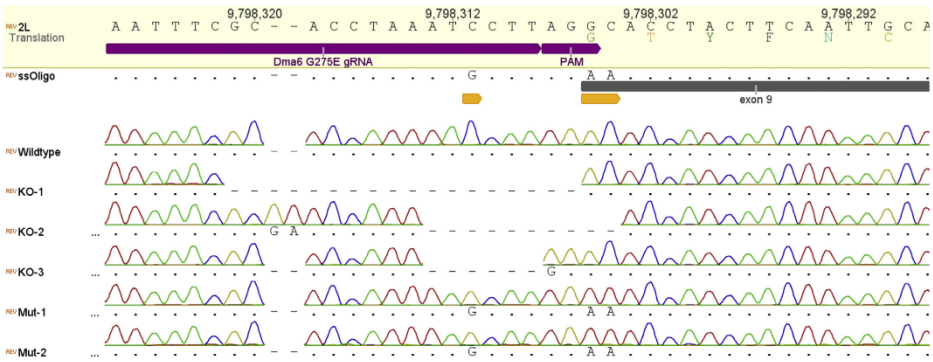

Fig. 2. Direct sequencing of Dα6 PCR fragments amplified from gDNA isolated from non-modified flies (wildtype), and spinosad resistant progeny after CRISPR (KO – knock outs, Mut – precise point mutation). The gRNA target site and exon 9 are annotated in purple and grey respectively. Point mutations are highlighted with orange arrows. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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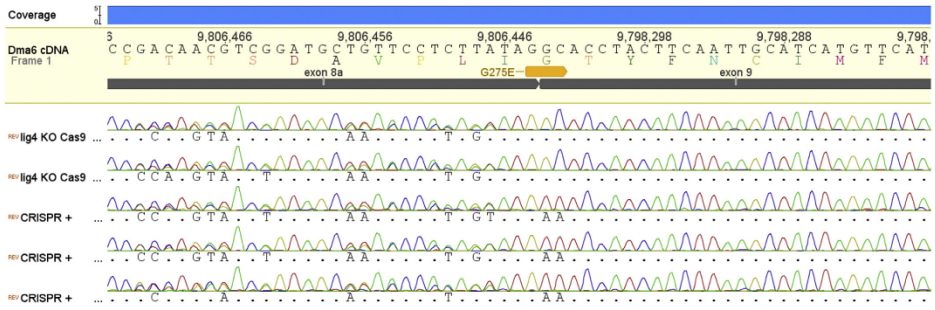

Fig. 3. Direct sequencing of Dα6 PCR fragments amplified from cDNA pools isolated from non-modified flies (lig4 KO Cas9), and flies carrying the G275E mutation (CRISPR−) codon position 901–903. Exons 8 and 9 are annotated in grey.

Table 2
Log-dose probit-mortality data for spinosad against *Drosophila melanogaster* strains and F1 progeny.

Strain/Genotype[a]	LC_{50} (mg/L^{-1})	95% CL	LC_{95} (mg/L^{-1})	95% CL	Slope (±SE)	Resistance ratio[b]		Dominance	
						LC_{50}	LC_{95}	LC_{50}	LC_{95}
Canton-S/+/+	5.7	4.92–7.12	10.04	8.5–12.6	4.254 (±0.279)	1	1		
lig4 KO Cas9/+/+	4.59	4.1–5.1	11.79	10.19–14.16	4.013 (±0.282)	0.8	1.2		
Dα6 G275E/G275E/G275E	354.8	322.93–398.58	665	565.2–827.1	6.029 (±0.499)	62.2	66.2	−0.951	−0.869
Dα6 G275E × Canton-S/G275E/+	22.04	16.39–29.6	85.76	57.08–171.2	2.787 (±0.409)	3.9	8.5	−0.951	−0.869
Dα6 G275E × lig4 KO Cas9/G275E/+	20.73	15.46–27.8	87.68	58.12–172.6	2.626 (±0.364)	3.6	8.7		
Dα6 KO −/−	791	678–923	3122	2453–4293	2.758 (±0.218)	138.8	310.9		
Dα6 KO × Canton-S/−/+	6.17	4.43–8.12	9.6	7.66–12.89	4.431 (±0.289)	1.1	1	−0.999	−1
Dα6 KO × lig4 KO Cas9/−/+	4.21	3.41–5.22	11.21	9.91–14.81	4.023 (±0.341)	0.7	1.1	−1	−1

[a] nAChR α6 – alleles (diploid) present in this strain, +/+ – homozygous wildtype, −/− – homozygous knock out, G275E/G275E – homozygous mutant.
[b] Resistance ratio is calculated by dividing LC_{50}/LC_{95} of any given strain with the LC_{50}/LC_{95} of Canton-S.

susceptible strains Canton-S and lig4 KO Cas9 confirmed the completely recessive inheritance of the Dα6 KO ($D = -1$) and revealed an incomplete recessive inheritance of the G275E mutation ($D = -0.951$ based on LC_{50} and $D = -0.869$ based on LC_{95}, Table 2).

4. Discussion

To date, the G275E mutation has been described in three different insect species (Bao et al., 2014; Puinean et al., 2013; Silva et al., 2016). However, recent studies on *F. occidentalis* strains from China and the USA have failed to detect any nAChR α6 sequence or expression differences between the susceptible and resistant strains prompting the authors to question the role of nAChR α6 subunit in spinosad resistance (Hou et al., 2014). Due to the acknowledged difficulties in expressing insect nAChR in heterologous systems, the role of G275E mutation in spinosad resistance was only inferred from studies done on surrogate receptors (Puinean et al., 2013). In order to establish whether G275E mutation in nAChR α6 subunit plays a direct role in spinosad resistance as suggested in previous studies (Bao et al., 2014; Puinean et al., 2013; Silva et al., 2016), we introduced the homologous mutation in *Drosophila* nAChR α6 receptor using the CRISPR/Cas9 system and compared the phenotypic resistance of mutated versus wild-type/background strain.

Genome editing using the CRISPR/Cas9 system has currently only been achieved in a handful of insect species, therefore we used *D. melanogaster* as a surrogate for those species known to carry a target-site mutation conferring spinosad resistance. We believe the use of *Drosophila* as a model in this context is entirely valid as a *Da6* orthologue is typically present in all insect genomes sequenced to date and is highly conserved (Jones and Sattelle, 2010, 2006; Shao et al., 2007) (Fig. 5). Moreover, it has also been shown that the role of the nAChR α6 in spinosad sensitivity is conserved across species, as α6 orthologues from different species are able to rescue a *Drosophila* spinosad resistant phenotype but not α5 or α7 which are closely related subunits (Perry et al., 2015).

Our results demonstrate that the introduced G275E mutation confers strong resistance in *Drosophila* to spinosad, with the LC_{50} increasing from around 5 mg L^{-1} to 335 mg L^{-1} (Fig. 4). The field rate of spinosad used for control of thrips and tomato leafminer is 87–120 mg L^{-1} and the level of resistance in *Drosophila* provides additional evidence that this mutation alone would be sufficient to result in control failure. Our finding that the G275E mutation is inherited as an incomplete recessive trait in *Drosophila* is in complete concordance with the mode of inheritance of spinosad resistance described in *F. occidentalis* (Bielza et al., 2007) where only homozygous females (or hemizygous males with a single copy of the resistance allele) exhibit sufficient resistance to survive field rates of spinosad. Interestingly, the level of resistance conferred by G275E (66-fold) was lower than for the *Drosophila* line with the *Dα6* null mutation (311-fold). As the nAChR α6 subunit is a redundant insecticide target it is not clear why loss-of-function mutations, which may confer higher resistance, are seen in certain spinosad resistant insect species and specific amino acid substitutions in others. While *Drosophila* α6 knock outs are viable

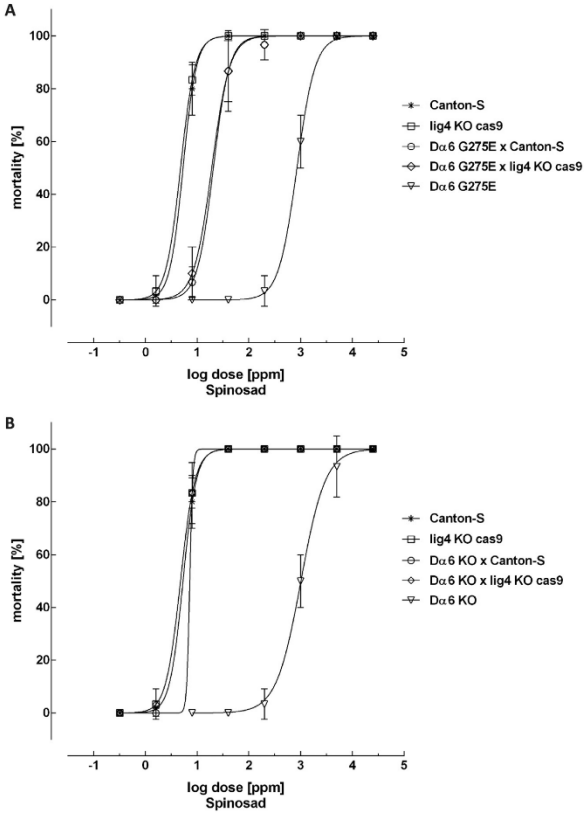

Fig. 4. Non-linear log dose-response plots for spinosad against *Drosophila melanogaster* strains and F1 progeny. Error bars represent standard deviation. A) Canton-S, lig4 KO Cas9, Dα6 G275E and F1 progeny of Dα6 G275E × Canton-S and Dα6 G275E × lig4 KO Cas9 respectively. B) Canton-S, lig4 KO Cas9, Dα6 KO and F1 progeny of Dα6 KO × Canton-S and Dα6 KO × lig4 KO Cas9 respectively.

and do not display an obvious fitness cost in the lab (Perry et al., 2007), it is likely that there is at least some reduction in fitness associated with loss-of-function mutations in the field environment. In this context a single amino acid substitution would be predicted to carry a lower fitness penalty and may be selected for over more profound genetic alterations in the *α6* gene, if such alterations confer sufficient resistance to survive exposure to recommended field rates. Interestingly, an additional point mutation associated with resistance has very recently been described in *D. melanogaster* where an amino acid replacement, P146S, was identified close to the conserved Cys-loop of the *Dα6* subunit and was shown to confer resistance using the CRISPR/Cas9 system (Somers et al., 2015). In comparison to loss of function mutations, point mutations can often provide new information on the mode and site of insecticide binding at the receptor (Bass et al., 2011; Troczka et al., 2012).

Although the precise location of the spinosad binding site is not known with certainty, homology modelling using the nematode glutamate-gated chloride (Glu-Cl) channel structure predicts the G275E mutation to lie at the top of the third α-helical transmembrane domain of the nAChR a6 subunit (Puinean et al., 2010). A recent crystal structure of the related Cys loop glutamate-gated chloride channel (GluCl) from *Caenorhabditis elegans*, co-crystallized with ivermectin – another macrocyclic lactone insecticide -, reveals that the corresponding amino acid is close (4.4 Å) to the binding site of ivermectin (Hibbs and Gouaux, 2011). In combination with the *in vitro* characterization of the modulatory effect of spinosad on human a7 nAChRs described in the introduction (Puinean et al., 2013) our work provides additional confirmation that the G275E mutation is directly involved in spinosad resistance.

Beyond this study we can see the utility of this model to principally investigate resistance mechanisms in a defined genetic

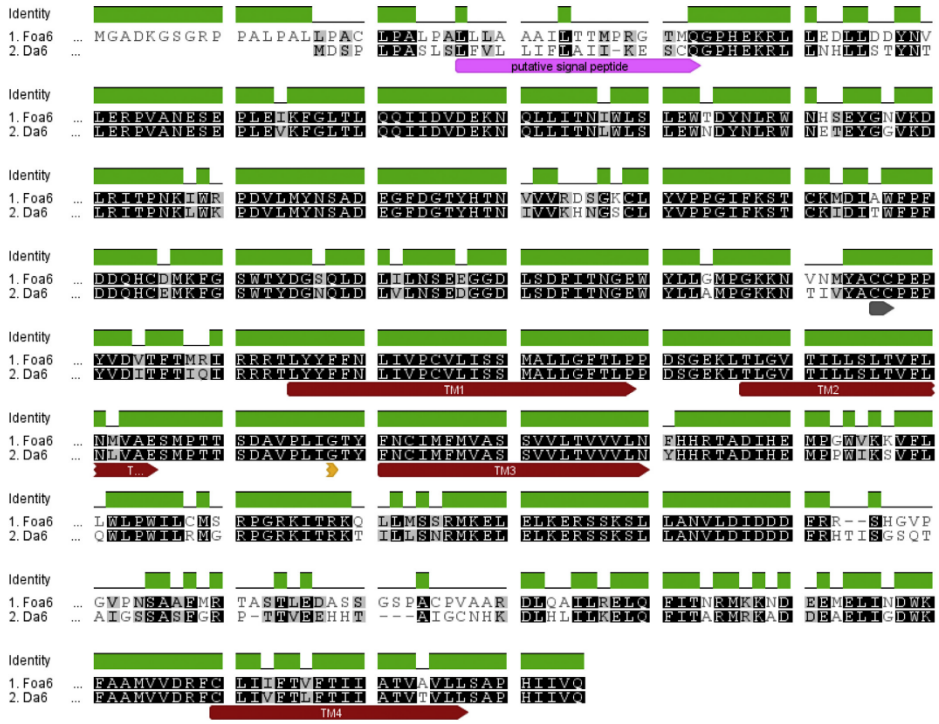

Fig. 5. Amino acid sequence alignment of *F. occidentalis* nAChR α6 and *D. melanogaster* nAChR α6 in variant exon 3b/8a. G275E is indicated by an orange arrow and counted from the first amino acid (G) after the putative signal peptide indicated by a pink arrow. Transmembrane regions TM1–4 are annotated with red arrows. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

setting. Functional validation of mutations identified in resistant insects is a crucial and yet often missing component of many studies, likely due, in part, to the difficulties involved in expressing insecticide target-sites *in vitro*. In this regard the CRISPR/Cas9 system applied to *Drosophila* is a straight forward and affordable approach to validate putative resistance mutations. Some caution is required as there is no guarantee that introducing SNPs in orthologous genes in *Drosophila* will resemble the exact phenotypes observed in the target organism. Success will likely be dependent on the location of the SNP in the gene of interest and the degree of conservation between orthologous genes of the target organism and the model species.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2016.04.007.

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